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PROTEIN AND AMINO ACID REQUIREMENTS OF MAMMALS

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Edited by

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Foreword

The past decade has witnessed a dramatic increase in public and scientific interest in nutrition. Because of the vital nature of protein in animal and human economy, a greater share of scientific attention has been directed to these food components than to any other moiety of the diet.

One of the major problems, if not the most important problem in nutrition, concerns the determination of the protein and amino acid requirements of various mammals, and man in particular. The approaches to this complex problem are numerous. It is the purpose of the volume to describe the techniques currently employed in this field, and to point out frankly their limitations. The reader will discover that although much has been accomplished, much more needs to be done before completely satisfactory values will be available on the protein and amino acid needs of mammals during growth, adulthood and senescence.

ANTHONY A. ALBANESE

New York, N. Y.
September, 1950

CONTENTS

	Page
Contributors	iv
Foreword	vii

Some Species and Age Differences in Amino Acid Requirements

By H. H. Mitchell, *Division of Animal Nutrition, University of Illinois, Urbana, Illinois*

I. Introduction	1
II. An Experimental Study	3
III. Keratin Synthesis in Protein Nutrition	9
IV. Composition of Tissue Proteins and Amino Acid Requirements for Growth	13
V. Amino Acid Requirements for Nitrogen Equilibrium in the Adult	18
VI. A Theory of Protein Metabolism	20
VII. A Schematic Representation of Protein Metabolism	25
VIII. Summary	28
References	29

Methods of Measuring the Nutritive Value of Protein Hydrolysates and Amino Acid Mixtures. The Rat Repletion Method

By Douglas V. Frost, *Abbott Laboratories, North Chicago, Illinois*

I. Introduction	34
II. Protein Evaluation Methods—The Older Methods	35
III. Assay Methods Based on Protein Regeneration	43
IV. Methods of Chemical Scoring	54
V. Specificity of Amino Acid Requirements	59
VI. Racemic Amino Acid Mixtures	67
VII. Summary	70
References	71

The Laboratory Evaluation of Amino Acid Mixtures and Protein Hydrolysates

By Robert H. Silber and Curt C. Porter, *Merck Institute for Therapeutic Research, Rahway, New Jersey*

I. Introduction	75
II. Experimental	77
III. Discussion	91
References	92

Dietary Proteins and Synthesis of Tissue Proteins

By Bacon F. Chow, *Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland*

I. Introduction 94
II. The Effect of Dietary Proteins on the Synthesis of Plasma Proteins . . 96
III. The Effect of Dietary Proteins on Repletion of Liver Proteins 107
IV. The Role of Dietary Proteins in the Hypertrophy of Gonads 108
V. Discussion 111
References 113

The Protein and Amino Acid Requirements of Man

By Anthony A. Albanese, *Nutritional Research Laboratory, St. Luke's Hospital, New York, New York*

I. Species Differences in Protein Needs 116
II. Qualitative Amino Acid Requirements of Various Species 118
III. Quantitative Amino Acid Requirements of Rat and Man 122
IV. Growth and Protein and Amino Acid Needs 127
V. Effect of Nutritional State on N-Requirements 128
VI. Metabolic Interactions of Essential and Nonessential Amino Acids . . 132
VII. Influence of Calories on N-Equilibrium and Amino Acid Requirements . 133
VIII. Influence of Vitamins on N-Metabolism 136
IX. Relation of Hormones, Proteins and Amino Acids 140
X. Summary 146
References 147
Index 153

Some Species and Age Differences in Amino Acid Requirements

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CONTENTS

I. Introduction 1
II. An Experimental Study 3
1. Experimental Methods 4
2. Experimental Results 4
3. Interpretations 5
4. A Test of the Amino Acid Adequacy of Whole Egg Proteins . . . 5
5. The Adult Rat Compared with Adult Man 7
III. Keratin Synthesis in Protein Nutrition 9
1. The Raiding of Protoplasmic Tissue for Methionine-Cystine 10
2. Wool Growth 11
3. Further Evidence and Recapitulation 12
IV. Composition of Tissue Proteins and Amino Acid Requirements for Growth 13
1. Similarities in the Amino Acid Content of Animal Tissue Proteins . 13
2. Biological Confirmation 15
3. Comparison with Growth Requirements 16
V. Amino Acid Requirements for Nitrogen Equilibrium in the Adult . . . 18
VI. A Theory of Protein Metabolism 20
1. Basic Principles 21
2. The Endogenous Protein Catabolism 22
3. Protein Stores in the Body 22
4. The Role of the Blood Plasma Proteins 23
5. Keratin Synthesis 23
6. The Exogenous Protein Catabolism Studied by the Isotope Technic . 24
VII. A Schematic Representation of Protein Metabolism 25
VIII. Summary 28
References 29

I. Introduction

The unique functions in the animal body served by the amino acids resulting from protein digestion are all anabolic in character. They relate to the replacement of essential tissue constituents that have been degraded in catabolic reactions, or to the formation of new tissue constituents in growth. In the rapidly growing animal, the latter functions dominate the body's requirements for amino acids. In the mature animal, the replacement functions may dominate the amino acid require-

ments, but the growth functions still persist, since some tissues continue to grow throughout life (1). Among adult animals of different species, the relative importance of the growth functions in determining amino acid requirements will depend mainly on the rate of growth of the epidermal structures, such as hair, nails and claws.

The different types of anabolic reactions occurring in the growing and in the adult organism with reference to the disposal of dietary amino acids lead inevitably to the conclusion that the amino acid requirements of infancy and adolescence, on the one hand, and of maturity, on the other hand, are different either in the assortment of amino acids that are required preformed in the diet, or in the relative amounts that are needed, or both. Species differences in amino acid requirements during growth would be expected not so much on the basis of differences in the nature of the anabolic reactions, as on the basis of differences in the rate of growth of new tissue in comparison with the weight of tissue to be maintained, that is, the percentage rate of growth. The higher the percentage growth rate, the greater the extent to which the growth of new tissue dominates the total amino acid requirements. The rat, growing at a rate of 3 per cent (or more) of its body weight daily, would need amino acids more in proportion to the needs for the formation of new tissues; the amino acid requirements of the human child growing at a rate of some 0.03 per cent of its body weight daily would be dominated more by the needs for amino acids to replace endogenous losses. In the former case, the daily requirement of protein, following the point of inflection in the growth curve, would be expected to decrease in absolute amount, as the daily growth increments decrease. In the latter case, the amount of protein required per day may be expected to increase as the body weight increases, but at a slower rate.

The amino acids needed for growth are needed mainly for the synthesis of the protein molecules entering into the structure of protoplasm. For this function the simultaneous presence in the tissues of all of the amino acids that the body cannot manufacture itself from dietary constituents is required. The absence of any one will block the synthetic processes. The amino acids needed for maintenance are needed mainly for the formation of creatine, carnosine, glutathione, ergothionine, thyroxine, adrenalin and other nitrogenous tissue constituents or tissue products destroyed in metabolism. For these replacement functions the assortment of amino acids needed is simple and will vary from one type of synthetic reaction to the other. The absence from the diet of any one essential amino acid will block one or more of these anabolic reactions but not all. Hence, a dietary protein like gelatin or zein, entirely deficient in one or more of the amino acids that the body cannot

manufacture itself, may be partially utilized in maintenance, as McCollum and Steenbock (2) showed many years ago in metabolism experiments on growing pigs. For growth, gelatin possesses a biological value of zero, since no growth will occur with gelatin as the sole source of amino acids. But for maintenance, it possesses a biological value of 25 (3), indicating that many of the replacement reactions of maintenance do not need the amino acid entirely lacking from the gelatin molecule, *i.e.*, tryptophane. Maintenance will also involve some protein synthesis for the growth of epidermal structures, for the replacement of red blood cells destroyed during metabolism, and probably for many other purposes, although the net aggregate in terms of nitrogen may not be large in proportion to the total replacement aggregate.

An important recent development in amino acid nutrition is the demonstration that the nonessential amino acids can be synthesized in the body of the monogastric animal from glutamic acid and the unnatural forms of the essential amino acids (4), from ammonium citrate (5), and from glycine and urea (6). The *ad libitum* feeding technique employed in the latter two reports detracts to some extent from the conclusiveness which the interpretation of the nitrogen-balance data might otherwise possess. The utilization of ammonia for amino acid synthesis (7, 8, 9), but not that of urea (10, 11), has been confirmed by the use of appropriate dietary supplements labeled with the N^{15} isotope.

II. An Experimental Study

The experiments that will be reported in this section were designed to test the statements just made concerning the differences in amino acid requirements that may be expected to accompany differences in age and in species among animals. They were carried out upon young and upon mature rats, not with different mixtures of amino acids, but with different proteins known to differ in amino acid content and in nutritive quality. The results obtained will be compared with those secured with adult human subjects subsisting on the same protein sources.

Most of the data on human subjects will be taken from an article published in 1948 from Murlin's laboratory at the University of Rochester (12). Many of the data on growing and adult rats have been taken from a paper by Mitchell and Beadles (13). Both series of experiments were part of a cooperative study of protein utilization sponsored by the Bureau of Biological Research of Rutgers University. The proteins, or protein foods, used by all cooperating laboratories were the same, consisting of egg albumin, desiccated and defatted whole egg, dried and defatted beef muscle, wheat gluten, casein and peanut flour.

1. Experimental Methods

The experiments, both those at the University of Illinois and those at the University of Rochester, were carried out in accordance with the nitrogen balance method, yielding coefficients of true digestibility and biological values. In the work on growing rats and in Murlin's work on adult men and women, these measures of protein utilization in digestion and in metabolism were computed by the Thomas method, or a modification of this method to adapt it to the growing animal. In the work on adult rats, the calculations were made on the basis of data secured with 3 dietary levels of each test protein; the biological values were computed from the slope of the line describing the relationship of absorbed nitrogen and of nitrogen balance, at levels of intake insufficient to support nitrogen equilibrium or to induce any considerable nitrogen retention (14), a modification of a method introduced by Melnick and Cowgill (15). The data were expressed per calorie of basal metabolism and were pooled together for each protein in computing regression equations. The standard errors of the slopes of the regression lines were computed by a method described by Rider (16).

2. Experimental Results

In Table I, the biological values of the nitrogen in the 6 protein sources for growing rats, for mature rats and for humans are assembled for comparison. The differences in digestibility, not given in the table, are not great with one or two exceptions and their discussion does not seem to be a profitable undertaking, since the factors determining the completeness of protein digestion are so little understood.

TABLE I

The Biological Value of the Test Proteins by Growing Rats, Mature Rats and Adult Men

Protein	Biological value		
	Growing rats	Mature rats	Mature humans
Egg albumin	97	94	91
Whole egg (commercial)	87	82	94
Beef muscle	76	69	67
Wheat gluten	40	65	42
Casein	69	51	56
Peanut flour	54	46	56

3. Interpretations

The differences in biological value, however, may be correlated to some extent with the amino acid composition of the proteins. Beef muscle, casein and peanut flour are deficient in cystine or methionine or both for the growing rat, while wheat gluten is markedly deficient in lysine (3).

From the table it will be evident that for those proteins deficient in cystine-methionine, *i.e.*, beef, casein and peanut, the biological values are lower for the adult rat than for the growing rat, and, with the exception of beef, they are lower than for the adult human. The cystine-methionine deficiency of beef muscle is comparatively small. On the other hand, for wheat gluten, deficient in lysine, the biological value for the adult rat is considerably higher than for the growing rat or for the adult human.

With regard to egg albumin and the proteins of whole egg, no such comparisons can be made among growing rats, mature rats and mature humans, because no conclusive information is available concerning their limiting amino acids. The estimations of Mitchell and Block (17), based upon the comparative amino acid analyses of whole egg proteins and of egg albumin would lead one to suspect a small lysine deficiency in the latter protein.

4. A Test of the Amino Acid Adequacy of Whole Egg Proteins

The nutritive quality of whole egg proteins and of egg albumin is so high that its limitation by amino acid deficiencies, if such exist, would be expected to be slight. If whole egg proteins are a perfect protein mixture for the growing rat, in the sense that they contain the essential amino acids in the same proportions that exist among the requirements for these amino acids, then supplementation of whole egg proteins with individual essential amino acids should not improve their growth-promoting value. In testing this proposition experimentally a whole egg sample prepared in the laboratory by fat extraction and dehydration at low temperatures was used. Samples prepared by this method have yielded biological values for the growing rat of 94 to 96, and protein efficiency ratios (in a 28-day test) averaging 4.00 g. weight gain per g. of protein consumed when fed at a dietary level of 9 to 10 per cent.

A basal diet was prepared containing approximately 9 per cent of conventional protein ($N \times 6.25$) from defatted dehydrated whole egg, 12 per cent of fat, 2 per cent of roughage and adequate additions of minerals and vitamins. It was fed for 28 days to 12 groups of 8 wean-

ling rats each to test the response in growth of supplements of individual amino acids, a mixture of 9 essential amino acids, omitting arginine, and of 3 per cent additional egg protein. Each group of rats was treated as 4 pairs, one rat in each pair receiving the unsupplemented basal diet and one receiving the respective supplement. The food intakes of pair mates were equalized. The average results of the test are summarized in Table II.

TABLE II

The Results of Amino Acid (or Protein) Supplementation of Laboratory-Prepared Whole-Egg Proteins Fed at a 9-Per Cent Level in Paired-Feeding Tests

Amino acid (or other) supplement	Concentration of supplement in diet %	Number of pairs	Average gain in body weight after 28 days of feeding: Without supplement, *g.*	With supplement, *g.*	Difference, *g.*
DL-Lysine	0.4	4	116.8	120.2	3.4
L-Histidine	0.2	4	91.7	92.7	1.0
L-Arginine	0.4	3[b]	74.3	76.0	1.7
DL-Phenylalanine	0.2	4	116.2	117.2	1.0
L-Tryptophane	0.2	4	107.5	108.0	0.5
DL-Methionine	0.2	4	115.0	116.2	1.2
DL-Threonine	0.2	4	88.5	86.5	—2.0
DL-Leucine	0.4	4	114.0	112.8	—1.2
DL-Isoleucine	0.6	3[b]	95.7	95.7	0
DL-Valine	0.2	4	117.8	121.5	3.7
9 essentials [a]	3.0	10	82.1	92.3	10.2
Additional egg proteins [a]	3.0	8	79.0	88.6	9.6

[a] These tests continued for only 21 days. In all pairs the rat on the supplemented diet gained at a faster rate and attained the greater body length.

[b] The group was reduced to 3 pairs because of the occurrence of respiratory disease in one rat of a pair.

The last two entries in the table demonstrate clearly that the 9 per cent level of egg protein was inadequate to promote maximum growth: in all pairs in each test the greater gain in body weight was made by the rat receiving the supplement of 3 per cent either of the 9 essential amino acids or of whole egg protein. The outcome of these tests prove that the basal diet was suitable for the detection of individual amino acid deficiencies.

The average growth responses induced by the individual amino acids were small, though in 7 of the 10 cases they favored the supplemented diet. If we consider the average differences between pair mates to be entirely due to uncontrolled experimental conditions, they can be pooled.

The mean of the pooled data is 0.95 g. favoring the supplemented diets, and the standard deviation of individual differences is 1.9. Since none of the mean differences in Table II exceeds twice this error, they may be considered in harmony with the initial assumption underlying this analysis, *i.e.*, that all differences in gains between paired rats are accountable on the basis of uncontrolled experimental conditions.

A separate consideration of the largest mean difference in Table II, *i.e.*, 3.7 g. for isoleucine, reveals that the variation among the 4 pairs in this comparison is such as to lead to a probability of 0.16 that the outcome was determined by a fortuitous combination of uncontrolled factors in the experiment. This probability is too large to be disregarded. For the next largest mean difference, 3.4 g. for the lysine test, a different situation exists. The 4 paired differences in this comparison are very uniform (4, 4, 3 and 3 g., all in favor of the supplemented diet), leading to a probability of only 0.0045 that the mean difference is entirely the result of the operation of random errors. Hence, this test proves, in so far as 4 replications of paired-feeding tests can prove, that the proteins of whole egg are definitely, if only slightly, deficient in lysine for maximum growth in young rats.

It is to be noted that the biological value for growing rats of the laboratory-prepared whole egg is considerably higher than that for the whole egg preparation included among the Rutger samples, *i.e.*, 94 compared with 87. This preparation must have undergone some nutritional damage during the processing to which it was subjected.

5. The Adult Rat Compared with Adult Man

The utilization of proteins in adult nutrition may be expressed, and compared among different species, as the amount of absorbed nitrogen per calorie of basal metabolism for nitrogen equilibrium. The justification for this method of expression is the Terroine-Smuts demonstration that the maintenance requirement of protein (nitrogen) in adult animals of different species varies with the basal metabolism (18), though it should be realized that the ratio of endogenous nitrogen loss to basal heat may be disturbed by differences in age, sex, prior treatment, etc. (19). The desired values may be obtained from the data of the experiment with mature rats from the regression equations of nitrogen balance on nitrogen absorbed, both being expressed per basal calorie, by solving for absorbed nitrogen intake when nitrogen balance is zero. The values for the adult human were computed in an analogous fashion from the data of Hawley and associates (12), supplemented by further necessary information kindly provided by Dr. J. R. Murlin. It should be noted

that the loss of nitrogen in the sweat of human subjects observed under sweating conditions is not ordinarily measured in nitrogen metabolism studies. On low-protein diets it may introduce an appreciable, but indeterminate, error in the computation of nitrogen balances and biological values (22).

The protein values expressed as milligrams of nitrogen per basal calorie required for nitrogen equilibrium, for mature rats and for mature humans, are listed in Table III. This table contains also data from some

TABLE III

The Requirements of Absorbed Nitrogen from Different Sources for Nitrogen Equilibrium by Adult Rats and Adult Humans

Protein source	Absorbed N required for N equilibrium	
	Adult rat	Adult human[a]
	mg. N per basal cal.	
I. Proteins of unknown deficiencies		
Whole egg, laboratory prepn.[b]	2.16	——
Whole egg, commercial	2.63	2.08
Egg albumin	2.30	2.14
II. Proteins deficient in cystine-methionine		
Beef muscle	3.11	2.93
Casein	4.24	3.31
Peanut flour	4.70	3.51
Soy flour	5.48	2.65
Milk	3.18	2.59
III. Proteins deficient in lysine		
Wheat gluten	3.20	4.67
Wheat flour	3.32[d]	4.61[c]

[a] Unless otherwise indicated, these data were derived from the values reported by Hawley *et al.* (12).

[b] This figure is the average excretion of nitrogen on a nitrogen-free diet of the adult rats in this experiment. Slightly deficient in lysine.

[c] The rat data are taken from Bricker and Mitchell (14), the data on humans from Bricker, Mitchell and Kinsman (20).

[d] Computed from a biological value of 65 (21) and the value of 2.16 mg. of nitrogen excreted per basal calorie on a nitrogen-free diet.

other sources than the two experiments heretofore considered. The values reveal again the more intense requirement of the adult human than of the adult rat for lysine, and the less intense requirement for methionine-cystine. If a protein is low in lysine more of it is required per basal calorie by man than by the rat, but if it is low in methionine-cystine, the reverse is true.

These results are in harmony with other experiments having a similar import. For example, Mueller and Cox (23) showed that casein and lactalbumin are equally effective in maintaining nitrogen balance in adult man. For the growing rat, the biological value of casein is much less than that for lactalbumin, 69 *vs.* 84 (24), but after cystine supplementation they are practically the same, 83 *vs.* 85. No similar comparison of casein and lactalbumin for the mature rat has been found in the literature.

Mitchell (21) observed no supplementing effect of lysine on the metabolic utilization of the proteins of white flour in the adult rat, from which he concluded on the basis of this and other pertinent evidence that "lysine is entirely dispensable in adult rodent nutrition, or is required in inconspicuous proportions for the maintenance of nitrogen equilibrium," in the sexually inactive rat. The latter reservation is inserted because of the finding of Pearson (25) that a lysine-deficient diet induces cessation of estrus cycles in the adult female rat. The latter alternative in the quotation is not inconsistent with the later work of Wissler and associates (26) in Cannon's laboratory. Contrast with these experiments of Mitchell (21) and especially the earlier ones by Osborne and Mendel cited by Mitchell, the one reported by Bricker, Mitchell and Kinsman (20), in which on a white-flour diet, an adult college woman was in marked negative nitrogen balance. When the diet was supplemented with lysine, the urinary nitrogen excretion per day dropped immediately by an amount equal to 3.5 times the nitrogen contained in the added lysine, and her nitrogen balance became positive. The need for lysine by the adult human had been previously shown by Rose (27) and by Albanese and coworkers (28). Of particular interest in this connection are the experiments of Hoffman and McNeil (29) showing with 10 hospital patients that the nitrogen balance index of Allison for the nitrogen of wheat gluten is raised from 0.62 to 0.76 by lysine supplementation.

III. Keratin Synthesis in Protein Nutrition

The relationships discussed above indicate that the cystine-methionine requirement is relatively more intense for the adult rat than for either the growing rat or for the adult human. On the other hand, the lysine requirement seems to be much less prominent among the amino acid requirements of the adult rat than among those of the growing rat or the adult human. How may these age and species differences be best explained?

The most probable explanation rests on the high cystine and low

lysine content of hair and other keratins (30) and on the probability that the amino acid requirements of the adult rat are dominated by the requirement for hair growth. The scleroproteins of hair contain 14 to 16 g. of cystine and less than 1 g. of methionine per 16 g. of nitrogen, and from 2 to 3.8 g. of lysine. The figures for lysine are similar to those reported for wheat gluten (2.0 g. per 16 g. of nitrogen) and for other cereal products. Miss Fraser (31) has shown that in the adult albino rat hair growth occurs continuously throughout life.

The importance of hair growth in determining the amino acid requirements for the growing rat would be much less than for the adult rat, since the most active protein growth relates to other tissues than hair. The cystine-methionine content of the proteins of these protoplasmic tissues is comparatively low, of the order of 4 to 6 g. per 16 g. of nitrogen, while the lysine content is high, 7 to 8 g. on the same basis. Hair growth in the adult human would also presumably be a minor factor in determining amino acid requirements since the human body is only sparsely covered with hair. This conception of the difference between the rat and man with respect to protein nutrition was stated by Mueller and Cox (23, 32).

1. The Raiding of Protoplasmic Tissues for Methionine-Cystine

The argument just presented assumes that hair growth will continue during periods when the animal is in negative nitrogen balance, since the protein utilization of adult rats and adult humans was determined under such conditions. Long periods of protein depletion will not denude a rat of its hair coat, probably because hair growth will continue at the expense of other tissue constituents. This transfer of amino acids from dispensable protein stores, using the term in the sense that Whipple has given it, may lead to an accelerated output of urinary nitrogen during specific protein inanition in proportion, in different animals, to the relative intensity of hair growth. The depression in the output of endogenous urinary nitrogen induced by the ingestion of methionine has been reported in the literature for rats (33) and dogs (34, 35), but not for man (36), may be due to the effect of this amino acid in arresting the raiding of protoplasmic tissues to secure cystine for hair growth where hair growth is a considerable item in the anabolic processes of the body.

An analogous situation was revealed by Ackerson and Blish (37) in their study of the effect of cystine on the endogenous metabolism of molting hens. For molting hens, in which feather replacement is proceeding at a rapid rate, the average daily output of nitrogen in feces and urine on a nitrogen-free diet was 239 mg. per kg. of body weight.

This value is considerably above the usual level of endogenous nitrogen excretion of mature hens. Another group of 6 hens of the same breed (Rhode Island Red) and size (about 2400 g.) received in addition to their nitrogen-free diet 150 mg. of cystine daily. The endogenous nitrogen loss in this group of birds averaged 137 mg. daily per kg. of body weight, a decrease of 43 per cent compared with the birds receiving no supplement. The decrease was highly significant statistically, and seemingly represents a sparing of an accelerated protein catabolism initiated by the necessity of securing cystine from other tissues for feather growth. However, the feeding of methionine to molting hens does not shorten the molting period (38).

2. Wool Growth

The persistence of keratin synthesis in animals under conditions of undernutrition is strikingly illustrated by experiments reported from the University of Illinois on wool growth in sheep. In experiments designed to measure the relative net energy value of alfalfa, clover and timothy hay for the maintenance of sheep, the consumption of hay was limited by the consumption of the least palatable one, namely, timothy hay. As a result 5 sheep during a feeding period of 200 days lost 20 per cent of their initial body weight (39). On analyzing their carcasses at the termination of this period and comparing the results with the composition of check sheep analyzed at the beginning of the experiment it was found that the undernourished sheep had lost 71 per cent of their fat, 48 per cent of their gross energy, but only 6.6 per cent of their protein, although they were in continuously negative nitrogen balance. However, the growth of wool, as well as its proximate analysis, was normal, amounting to 0.134 lb. of protein and 633 cals. of gross energy per day per 1000 lbs. live weight. A total of 961 g. of protein had been laid down in the fleece. Much of this keratin nitrogen must have been produced by raiding the protoplasmic tissues for the necessary amino acids, particularly for cystine and methionine.

The relative dominance of wool growth in the protein nutrition of young, growing lambs and of more mature animals is shown by two other experiments from the same laboratory (40, 41), carried out in much the same way except that supermaintenance, rather than submaintenance rations, were fed. In one experiment, 9 lambs weighing 65 to 70 lbs. initially were fed for 131 to 141 days on a ration of alfalfa hay and corn. Their average daily gains in body weight ranged from 0.18 to 0.36 lb. On carcass analysis, wool growth was found to account for 26 per cent of the retained nitrogen. A second group of more mature

lambs, weighing initially 84 to 91 lbs., were fed on alfalfa hay alone for 182 days, making average daily gains in body weight of 0.10 to 0.23 lb. Of the protein stored during this period, an average of 60 per cent was recovered in the wool growth.[1]

3. Further Evidence and Recapitulation

Thus, the theory that explains best the different results on protein utilization secured with growing and mature rats and with human adults is that the relative dominance of hair growth in the protein metabolism of animals may, in the main, determine the proportionate requirements of the various amino acids, because of the peculiar amino acid composition of the scleroproteins. Their richness in cystine would intensify the need for the sulfur-containing amino acids to the extent that the demands of amino acids for hair growth dominate the demands for protoplasmic growth and for endogenous replacements. The richness of the keratins of feathers in arginine seems to be responsible for the accentuated demands of the chicken for this amino acid (42), the arginine requirement being greater for the more rapidly feathering breeds. The poverty of hair proteins in lysine would depress the proportionate lysine requirements. These predictions are borne out by the data presented. Keratins are low in histidine, 1.0 g. per 16 g. of nitrogen, and in phenylalanine, 3.7 g. per 16 g. of nitrogen, in comparison with whole egg protein. If the theory is correct, one would expect that the histidine and phenylalanine requirements of an animal in which the protein anabolism is dominated by hair growth (keratin synthesis) would be inconspicuous. Burroughs, Burroughs and Mitchell (43) were able to maintain adult rats in nitrogen equilibrium for short periods on diets lacking in lysine, histidine, and phenylalanine if tyrosine was present.

The peculiar amino acid composition of keratins in hair, nails, wool, feathers, etc., and the relative magnitude of keratin synthesis, which persists throughout life, may well be the most important factors in determining differences in the proportionate amino acid requirements of animals of different species and ages.

[1] Of the same significance are the observations of Peirce (*Council for Sci. and Indust. Res., Australia, Bul.* **121.** 1938) that fluorine concentrations in the ration of sheep that ultimately induce serious loss in body weight have no appreciable effect on wool growth.

IV. Composition of Tissue Proteins and Amino Acid Requirements for Growth

A logical extension of the conception stated in the last section is that the amino acid requirements of the growing animal are determined in the last analysis by the amino acid composition of the tissue proteins formed during growth. While in the adult animal the amino acid content of the keratin tissues constituting the integument and its appendages seems to dominate the total amino acid requirements in accordance with the relative intensity of the growth of hair, wool, feathers, etc., as compared with the replacement of the endogenous losses of nitrogenous material, in the young animal, and particularly the rapidly growing animal, the amino acid content of the protoplasmic tissues would be expected to dominate the picture. The amino acid analyses of groups of animal tissues assembled in Table IV should afford a test of this hypothesis.

1. Similarities in the Amino Acid Content of Animal Tissue Proteins

The amino acid content of muscle tissue among mammalian forms of life is strikingly similar (3), and hence the analyses of muscles from different species have been pooled in Table IV. In fact, quoting from Beach, Munks and Robinson (44): "The protein mixture which makes up voluntary muscle tissues is similar in Mammalia, Aves, Amphibia, Pisces and Crustacea, with respect to ten of the amino acids. Since muscle tissues of these various classes of animals do not differ widely in their amino acid patterns, the findings support the belief that the same or closely similar amino acid composition of muscle proteins is repeated throughout the animal kingdom."

Among the visceral organs, the liver, kidney and brain are quite similar in amino acid composition, differing from lung and stomach in higher contents of tryptophane, cystine-methionine, and phenylalanine-tyrosine. As a class, the proteins of the visceral organs differ from muscle proteins chiefly in a lower content of lysine.

The blood plasma proteins, to which Whipple (47) assigns such a prominent role in the protein exchanges of the body, are distinguished from muscle proteins mainly by a larger content of lysine, tryptophane, methionine-cystine, and leucine and a much lower content of isoleucine.

The keratins, as noted earlier, have their own peculiar amino acid pattern, characterized mainly by their deficiency in lysine, histidine, and phenylalanine, and their excessive contents of arginine but particularly of cystine. Collagen and elastin, the proteins of connective tissue, car-

TABLE IV

Amino Acid Composition of Animal Tissue Proteins Expressed in Grams per 16 Grams of Nitrogen

Amino acid	Mammalian muscle proteins		Visceral organ proteins		Blood plasma proteins	Keratins		Connective tissue proteins	
	[a]	[b]	[c]	[d]	[e]	Average [f]	Hog hair [g]	Collagen [h]	Elastin [h]
Lysine	8.1	8.6	6.2	6.0	9.6	3.2	3.8	4.8	0.40
Histidine	2.9	2.3	2.9	2.1	3.1	1.0	1.1	0.62	0.10
Arginine	7.1	7.1	6.5	6.6	6.2	10.7	10.9	7.9	0.88
Phenylalanine	5.1	4.4	5.8	5.0	5.9	3.7	2.7	2.1	5.0
Tyrosine	4.1	4.5	4.5	4.4	5.4	5.1	3.5	0.84	2.0
Tryptophane	1.3	1.3	1.6	1.5	1.8	1.4	—	<0.01	<0.01
Methionine	3.2	3.4	3.0	2.8	2.0	1.0	0.5	0.89	0.06
Cystine	1.2	0.9	1.6	1.1	3.6	10-17	14.4	0.09	0.16
Threonine	4.6	4.8	5.1	4.5	6.4	7.2	6.3	2.2	1.1
Leucine	8.0	—	7.9	—	17.8	10.0	8.3	3.0	7.9
Isoleucine	5.9	—	5.4	—	3.1	5.0	4.7	1.7	3.5
Valine	6.0	—	5.4	—	6.0	6.0	5.9	2.4	16.3

[a] Beef, horse, chicken, beef heart (3).

[b] Beef, veal, lamb, pork, chicken (44).

[c] Liver, kidney, brain (3).

[d] Liver, kidney, brain, lung, stomach (44).

[e] Computed from the data given by Mitchell and Block (17) for serum proteins and fibrin, assuming that fibrinogen has the same amino acid composition as fibrin and constitutes 4 per cent of the total plasma proteins.

[f] (3).

[g] Results expressed on water- and fat-free basis (45).

[h] Averages of 12 samples of collagen and 6 samples of elastin from different sources (46).

tilage and bone are even more unique in their amino acid composition. They are unusually rich in glycine and in proline (46).

2. Biological Confirmation

The similarity of the essential amino acid contents of muscle proteins and visceral proteins, regardless of species, is clearly indicated by the similarity in their biological values for growing rats. The following biological values have been reported from various laboratories (see 3): beef muscle 76, pork tenderloin 79, ham 74, red salmon 73, beef heart 74, beef liver 77, and beef kidney 77. Further evidence to the same effect is summarized in Table V, which contains protein efficiency ratios (grams

TABLE V

Nutritive Value of the Proteins from Muscles and Visceral Organs of Different Animals, as Measured by the Protein Efficiency Ratio with Growing Rats [a]

Tissue and animal	Protein efficiency ratio	Tissue and animal	Protein efficiency ratio
Muscle:		Liver:	
Ox, mature	3.08	Ox	2.67
Ox, immature	2.88	Hog	3.21
Hog	2.79	Sheep	3.00
Sheep	2.94	Kidney:	
Ox heart	3.10	Ox	2.86
Hog heart	2.91	Hog	3.04
Ox tongue	2.85	Brain:	
Hog tongue	2.86	Ox	2.97
Spleen:		Hog	2.89
Ox	2.75		

[a] Taken from Hoagland and Snider (48, 59).

gain in weight per g. of protein consumed) for many types of muscle and visceral organs and for several species. These ratios are comparable, since they were obtained under standardized conditions in the laboratory of Hoagland (48, 49). They all fall within the range 2.75 to 3.21. However, in muscular tissues containing appreciable proportions of connective tissue (50), the nutritional value of the combined proteins may be quite low (51), due to the unique amino acid deficiencies of collagen and elastin.

3. Comparison with Growth Requirements

Since the muscles and the visceral organs contain most of the proteins in the animal body, the proportions of the essential amino acids contained in these proteins should largely determine the proportions among the requirements for these acids by the growing animal on the basis of the thesis under discussion. A test of this proposition is afforded by the data given in Table VI, in which amounts of amino acids in the tissues or amounts estimated to be required are all computed using lysine as a base with a value of 100. Analogous values for whole egg proteins (3) are included also, representing a dietary protein very completely utilized in metabolism.

A close correlation between tissue composition and amino acid requirements may not be revealed by the values given in Table VI because

TABLE VI

Relative Proportions Among the Essential Amino Acids in Animal Tissues and in Whole Egg as Compared with Estimated Requirements.
Lysine is Given a Value of 100

				Estimated requirements	
Amino acid	Muscle	Visceral organs	Whole egg	Rat [a]	Chick [b]
Lysine	100	100	100	100	100
Histidine	36	47	29	40	33
Arginine	88	105	90	20	133
Phenylalanine + tyrosine	114	166	151	90	156 [c]
Tryptophane	16	26	21	20	20 [d]
Methionine + cystine	55	74	90	60	100
Threonine	57	82	68	50	50 [e]
Leucine	99	127	128	80	156
Isoleucine	73	87	111	50	56
Valine	74	87	101	70	78

[a] Rose (6). The unconverted lysine requirement is 1.0 per cent of the diet.

[b] Taken from Almquist (52) except where otherwise indicated. The unconverted lysine requirement is 0.9 per cent of the diet.

[c] Grau (53).

[d] Wilkening *et al.* (54).

[e] Grau (55).

of errors in amino acid analyses, but particularly because of uncertainties in estimates of amino acid requirements. Rose's values for the rat, originally proclaimed in 1937 (56) and revised slightly in 1949 (6) are considered by their author as tentative; their evidential support has

never been revealed. The amino acid requirements of the chick may not be entirely satisfactory, because of the subnormal growth generally secured by the *ad libitum* feeding of amino acid mixtures in lieu of protein, and because the interpretation of the data is sometimes obscure. Nevertheless, a high correlation is evident between the amino acids in muscle and the estimates of amino acid needs of the growing rat, if the arginine values are disregarded because of the limited ability of the rat to synthesize this amino acid. The product-moment correlation coefficient is +0.94. The correlation between the amino acid contents of visceral organs and the Rose estimates is also high, with r = +0.83. The chick requirements for the essential amino acids, including arginine, are also highly correlated with the essential amino acids of muscle tissue (r = 0.87). Note the high requirements of the chick for methionine-cystine and arginine, as compared with the rat, due in all probability to a more rapid keratin synthesis in feather growth.

These and other correlation coefficients among the variates in Table VI are assembled in Table VII. The proteins of whole egg, which are

TABLE VII

Correlations Among Variates in Table VI: Product-Moment Correlation Coefficients

	Covariates				
		Amino acids in		Amino acids required by	
Variates	muscle	organs	whole egg	rats	chickens
Amino acids in					
muscle	—	0.67	0.92	0.94[a]	0.87
organs	0.67	—	0.70	0.83[a]	0.80
whole egg	0.92	0.70	—	0.81[a]	0.83

[a] Omitting the arginine values in both variates.

so highly utilized in metabolism, are highly correlated in their content of the essential amino acids with the proteins of muscle (r = 0.92), for which they serve so effectively as dietary precursors.

The evidence presented in Tables V, VI, and VII proves that the requirements of the essential amino acids that must be provided preformed in the diet of the growing animal are determined to a very large extent, probably to a larger extent than the cited evidence indicates, by the amino acid makeup of the tissues they are producing. This fact suggests that an effective method of determining the requirements of a growing animal for these amino acids may be to determine, first, the requirement in grams per day of some one amino acid, such as lysine, and then to estimate the requirements of the others from the proportion

existing between the essential amino acids and lysine in the body of the animal, these proportions to be determined by amino acid assays of the entire carcass, or, approximately, even by amino acid assays of a dominant tissue such as muscle. This method may be as accurate as methods in current use for measuring amino acid requirements. It has the distinct advantage of avoiding the confusion concerning the utilization of the D-isomers that are commonly fed in racemic mixtures. The usual assumption that the utilization of these isomers is an "all or none" proposition has been shown to be wrong, in some instances at least, since partial utilization has been established (56, 57, 58).

V. Amino Acid Requirements for Nitrogen Equilibrium in the Adult

Estimations of the amino acid requirements of the adult organism are not in a satisfactory state, even as regards the number of amino acids required. The validity of the use of nitrogen equilibrium as a criterion of amino acid adequacy has been challenged (60, 61), particularly in the case of arginine and of histidine (62). Certainly such a criterion fails to consider the adult accretion of protein material in the growth of those tissues, mainly the integumental structures, that grow throughout life. For adult man, the nitrogen equivalent of "adult growth" has been assessed at about 0.56 g. per m^2 of body surface daily (1). In evaluating nitrogen balance studies the dermal loss of nitrogen must also be considered, although it rarely is. Insensible perspiration contains appreciable quantities of nitrogen, 180 to 230 mg. per day, according to Freyberg and Grant (63). Under minimal sweating conditions, this dermal loss may amount to 0.36 g. of nitrogen daily (22). When sweating occurs, whether psychic or thermic in origin, the loss of nitrogen (22, 64) and of amino acids (65) will be greatly increased.

Thus, amino acid requirements in adult animals assessed by nitrogen balance studies are minimal figures, but they may serve the purpose of comparing the magnitudes of the requirements of the different essential amino acids, using this term to cover the 10 amino acids originally shown by Rose to be essential for normal growth. The experiments of Burroughs, Burroughs and Mitchell (43) indicate that the requirements of the adult rat for lysine, histidine, and phenylalanine are inconspicuous in magnitude, probably because of the dominance of keratin synthesis in the protein nutrition of the adult rat.

The values given in Table III on the absorbed nitrogen per basal calorie from different proteins and protein foods required for nitrogen equilibrium in the adult rat and in adult man, together with the deter-

mined contents of these protein foods in the essential amino acids per 16 g. of nitrogen (3) have been used to compute the daily requirements of the individual amino acids for a 325 g. rat and 70 kg. man. For each protein for which a requirement is given in Table III, the corresponding amounts of the different essential amino acids were listed, both for the rat and for man. The required amount for each amino acid in each case is then taken as the smallest amount in the list. The requirements thus obtained are summarized in Table VIII.[2]

TABLE VIII

Estimated Daily Amino Acid Requirements for the Adult Rat [a] and the Adult Man [b]

Amino acids	Estimated requirements for the rat [c]	Requirements for adult man	
		Estimated [c]	Reported by Rose (66)
	mg.	*g.*	*g.*
Arginine	21.9	1.18	0
Histidine	7.8	0.55	0
Lysine	11.2	0.99	0.80
Phenylalanine-tyrosine	40.3	2.42	1.10
Tryptophane	5.6 [d]	0.32	0.25
Methionine-cystine	19.1	1.05	1.10
Threonine	12.2	0.56	0.50
Leucine	34.3	1.86	1.10
Isoleucine	20.8	1.12	0.70
Valine	23.6	1.18	0.80

[a] For male albino rat weighing 325 g. with a basal metabolism of 27.6 cals. per day.

[b] For a man, 70 kg. in weight, 174 cm. in height, 25 years of age, with a basal metabolism of 1700 cals. per day.

[c] Based upon data in Table III and the amino acid composition of the respective proteins (3), as explained in the text.

[d] Estimated from nitrogen balance studies at 4.0 to 6.4 mg. for a 200 g. rat by Wykes, Henderson and Elvehjem (*J. Nutrition*, 40, 71. 1950).

The requirements for adult man are listed side by side with those recently proclaimed by Rose (66), for which supporting evidence has not been offered. The two may not be comparable, first, because of the indirect method of estimation used to secure the values given in column 3 of the table, and, second, because Rose's values, although described

[2] Essentially the same method was used by Harte and Travers (*Science*, 105, 15. 1947) in estimating the amino acid requirements for adult man from less extensive data.

as "minimum daily requirements," are not averages of the 8 to 31 individual determinations for the different amino acids, and hence are not the most probable requirements for any given individual or group of individuals. They are the highest of the group of determinations in each case. According to Rose: "Furthermore, when several subjects show different requirements for a given amino acid, the highest level must be taken as the minimum. Any 'recommended intakes' must make further allowances for the possibility that certain individuals may need still larger quantities than the subjects thus far tested." It would be expected that the range between the most probable requirement (the average) and the highest requirement (the one selected) would be the greater, the larger the group of subjects tested. Until the individual data are reported, it is impossible to evaluate adequately these experiments of Rose.

The estimated values given in Table VIII would be expected, from the method of their calculation, to be nearer the minimum values for those amino acids in which the basic proteins are deficient, *i.e.*, for lysine and for methionine-cystine. The values for the other amino acids may be larger than the true requirement, either because they are contained in all of the basic proteins in amounts larger than those required, or because the amounts derived by the method employed include certain fractions used for the synthesis of the non-essential amino acids. It may be more of a coincidence than otherwise that the estimated human requirements for lysine and methionine-cystine, as well as those for tryptophane and threonine, are of the same order of magnitude as the "minimum requirements" announced by Rose.

Finally, it may be noted from the estimated requirements given in columns 2 and 3 that the ratio of the methionine-cystine to the lysine requirement is much greater for the adult rat than for the adult human, *i.e.*, 1.71 and 1.06, respectively. This relationship conforms with expectation in view of the probable greater dominance of keratin synthesis in the protein nutrition of the adult rat than in that of the adult human.

VI. A Theory of Protein Metabolism

An important step in the development of a science is the incorporation of new ideas and facts into its body of laws and principles. Of no less importance is the harmonizing of conflicting theories and interpretations of facts. The facts, if they are such, cannot be in conflict.

The present status of the theories of protein metabolism is not a harmonious one. A rediscussion of the problem is therefore in order.

1. Basic Principles

The experimental investigations of Folin on the effect of the protein level of the diet on the composition of human urine (67) mark the beginning of modern concepts of protein nutrition. The facts thus established are still valid. Their interpretation by Folin (68) was a logical one, and to a large degree the essential points of this interpretation are valid today and can successfully withstand criticism. The basis of Folin's theory of protein metabolism can best be presented by a few quotations from his classical paper (68).

"We have seen from the tables that the composition of urine, representing 15 gm. of nitrogen, or about 95 gm. of protein, differs very widely from the composition of urine representing only 3 gm. or 4 gm. of nitrogen, and that there is a gradual and regular transition from the one to the other. To explain such changes in the composition of the urine on the basis of protein katabolism, we are forced, it seems to me, to assume that katabolism is not all of one kind. There must be at least two kinds. Moreover, from the nature of the changes in the distribution of the urinary constituents, it can be affirmed, I think, that the two forms of protein katabolism are essentially independent and quite different. One kind is extremely variable in quantity, the other tends to remain constant. The one kind yields chiefly urea and inorganic sulphates, no kreatinin and probably no neutral sulphur. The other, the constant katabolism, is largely represented by kreatinin and neutral sulphur, and to a less extent by uric acid and ethereal sulphates. The more the total katabolism is reduced, the more prominent become these representatives of the constant katabolism, the less prominent become the two chief representatives of the variable katabolism."

"The fact that the kreatinin elimination is not diminished when practically no protein is furnished with the food, and that the elimination of some of the other constituents is only a little reduced under such conditions, shows why a certain amount of protein must be furnished with the food if nitrogen equilibrium is to be maintained. It is clear that the metabolic processes resulting in the end products which tend to be constant in quantity appear to be indispensable for the continuation of life; or, to be more definite, those metabolic processes probably constitute an essential part of the activity which distinguishes living cells from dead ones. I would therefore call the protein metabolism which tends to be constant, *tissue* metabolism or *endogenous* metabolism, and the other, the variable protein metabolism, I would call the *exogenous* or intermediate metabolism."

"The exogenous or intermediate protein katabolism is here con-

ceived as consisting of a series of hydrolytic splittings resulting in a rapid elimination of the protein-nitrogen as urea.''

Later advances in our knowledge of protein metabolism relate mainly to the details of the processes of protein assimilation and protein utilization. The main picture as outlined by Folin in 1905 remains essentially unchanged.

2. The Endogenous Protein Catabolism

The essential constancy of the endogenous protein catabolism has been repeatedly confirmed and its approximately constant relationship to the basal energy metabolism has been established (13 and literature there cited; 69, 70), as well as the conditions disturbing this relationship (71, 19). The independence of the endogenous and the exogenous metabolism of nitrogen was confirmed experimentally by Burroughs, Burroughs and Mitchell (72).

Of particular significance is the work of Schoenheimer's group on the metabolism of creatine using the isotope technic. They showed (73) that muscle creatine is dehydrated to creatinine at a very constant rate, and ''is not involved in any biological reaction in which linkages between carbon and carbon and carbon and nitrogen are broken.'' ''It thus differs in its metabolic aspects from all other biological compounds so far investigated with isotopes.''

3. Protein Stores in the Body

Whipple and his group have established the existence of dispensable and indispensable stores of protein in the animal body (47) in the course of their work on hemoglobin and blood plasma protein regeneration. Dispensable stores are readily raided when emergency demands for specific amino acids or for protein are not covered by the food supply. The forms in which these protein stores exist is not known. Their ready mobility apparently depends upon their exposed position in the cells rather than upon their chemical nature; an analogy would be the ready accessibility of the calcium salts in the epiphysis, as contrasted with the diaphysis, of the long bones (74). While the location of the protein stores is probably quite general, the outstanding role of the liver in the capacity of a storage organ seems clear (75, 76, 77).

The dispensable protein stores rise and fall as the protein intake rises or falls. While these stores appear to contribute nothing to the normal functioning of the body cells, they do exert a protective function when the body is subjected to the strain of chloroform or arsphenamine poisoning, and other destructive agencies, and, in Whipple's words, they

are "a bulwark against infection." They bear the brunt of sudden increases in protein catabolism due to inflammation, hyperthyroidism, and probably other stresses.

4. The Role of the Blood Plasma Proteins

Before it was shown experimentally that the amino acids resulting from protein digestion gained access to the blood as such, Abderhalden (78) proposed the theory that, in their passage through the intestinal mucosa, the proteinogenous amino acids were combined to form the blood plasma proteins which in turn nourished the cells of the body. The theory served a good purpose in explaining the facts available at the time it was elaborated. It was rightly discarded when the occurrence of hyper-aminoacidemia during protein digestion was established.

It is a matter of considerable historical interest that the theory in some of its features has been revived, mainly as a result of the work of Whipple and his associates (47). The ability of the blood plasma proteins to nourish the other cells of the body is evident from the fact that an animal may be kept in nitrogen equilibrium by the intravenous injection of compatible blood plasma as the sole source of nitrogen (79). The conversion of blood plasma protein into cytoplasmic protein occurs apparently without prior degradation into amino acids, since phlorizinized dogs thus nourished do not excrete extra sugar in the urine. According to Howland and Hawkins (80):

"The metabolism of protein when fed is different than when it is injected. It is suggested that there is a partial catabolism of the injected protein with reassembly of the large aggregates formed by the cells to form their own peculiar type of protein.

"This partial catabolism with reassembly of the large aggregates may be the method of protein interchange within the body rather than a complete catabolism to amino acids with subsequent resynthesis to protein."

Reineke, Williamson and Turner (81) provide additional evidence of this type of protein interchange in their demonstration that milk globulin is formed in the mammary gland of the goat from a globulin, glycoprotein, of the blood plasma.

5. Keratin Synthesis

The peculiar amino acid demands for keratin synthesis, due to the unique amino acid composition of keratins, together with the persistence of this synthesis under conditions of protein under-nutrition, at the

expense of protoplasmic proteins in other tissues, has been fully established and discussed above. Its dominance in the protein nutrition of maturity in those animals supporting a full coat of hair, wool, fur or feathers is to be expected.

6. The Exogenous Protein Catabolism Studied by the Isotope Technic

The investigations of Schoenheimer and his group at Columbia University on intermediary protein metabolism using amino acids labeled with N^{15} have revolutionized conceptions of the exogenous metabolism of Folin. They have revealed a dynamic, rather than a static, state of the tissue proteins (82), and have shown "that all reactions for which specific enzymes and substrates exist in the animal, are carried out continuously." Quoting further from the writings of this group (83):

"It has been shown that nitrogenous groupings of tissue proteins are constantly involved in chemical reactions; peptide linkages open, the amino acids liberated mix with others of the same species of whatever source, diet, or tissue. This mixture of amino acid molecules, while in the free state, takes part in a variety of chemical reactions: some reenter directly into vacant positions left open by the rupture of peptide linkages; others transfer their nitrogen to deaminated molecules to form new amino acids. These in turn continuously enter the same chemical cycles which render the source of the nitrogen indistinguishable. Some body constituents like glutamic and aspartic acids and some proteins like those of liver, serum, and other organs are more actively involved than others in this general metabolic mixing process. The excreted nitrogen may be considered as a part of the metabolic pool originating from interaction of dietary nitrogen with the relatively large quantities of reactive tissue nitrogen."

Thus, our knowledge of the nature of intermediary protein metabolism has undergone a profound change since the time of Folin. But as far as end results are concerned, these isotope studies have not changed the Folin conception of the exogenous metabolism in the slightest. These reversible reactions revealed by isotope studies between tissue proteins and dietary amino acids are not anarchistic in nature. "They seem . . . to represent automatic and non-interruptable biochemical processes, of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change" (84). Hence, for all practical purposes the tissue proteins may be considered to be static.

Furthermore, the magnitude of these intermediary reactions, and in particular the amount of nitrogen excreted in the urine as a result, is

determined primarily by the magnitude of the nitrogen intake of the animal. Urea and ammonia are still the primary end-products that appear in the urine. These reversible reactions, which may, according to Sprinson and Rittenberg (85), involve only a fraction of the muscle proteins, possibly only dispensable protein in the Whipple sense of the term, are sharply distinguished from the irreversible reactions involving tissue proteins and other nitrogenous constituents, characteristic of the endogenous catabolism of Folin and typified by the creatine dehydration to creatinine.

The main effect of these isotope studies of the intermediary protein metabolism is to render the term "exogenous" somewhat, though not completely, inappropriate. It is still the body's method of ridding itself of nitrogen consumed in amounts exceeding its needs for endogenous replacement and for growth, nitrogen being the one element in the protein molecule that it cannot oxidize.

VII. A Schematic Representation of Protein Metabolism

Fig. 1 is an attempt to combine into one picture the facts and the modern theories of protein metabolism and protein assimilation. The diagram consists of a series of cells bearing labels possessing a biochemical or functional, rather than an anatomical significance, grouped about the metabolic pool of amino acids which has been recently defined by Sprinson and Rittenberg (85) in the following terms:

"We shall here define the metabolic pool of the animal (or organ or cell) as that mixture of compounds, derived either from the diet or from the breakdown of the tissues, which the animal (or organ or cell) employs for the synthesis of tissue constituents. The nitrogenous compounds of the metabolic pool constitute the nitrogen pool.

"It is probable that all of the constituents of the nitrogen pool are present in the non-protein fraction. The reverse is not true. Urea and creatinine, for example, are not part of the metabolic pool, since they are solely excretory products . . ."

The flow lines identified by numbers in circles originate with the "dietary protein" cell at the top of the diagram and may be described briefly as follows:

(1) Incomplete digestion of dietary protein.

(2) Gastrointestinal proteolysis, absorption into the blood and transportation to the tissues where the resulting amino acids are incorporated into the metabolic pool.

(3) Loss of body nitrogen in the feces, the so-called metabolic fecal nitrogen. This nitrogen originates within the body of the animal and

is contained in the unabsorbed constituents of the digestive juices, and cellular material (86) and mucus derived from the gastric and intestinal mucosa. Its significance and importance in ruminant nutrition have been discussed by Blaxter and Mitchell (87). Its replacement is essential to the maintenance of the nitrogenous integrity of the body and it is hence an item in the protein requirement of the animal. That this replacement occurs under normal nutritional conditions is clearly shown by the observations of Tarver and Schmidt (88) and of Friedberg (89) using dietary amino acids labeled with radioactive isotopes of sulfur or

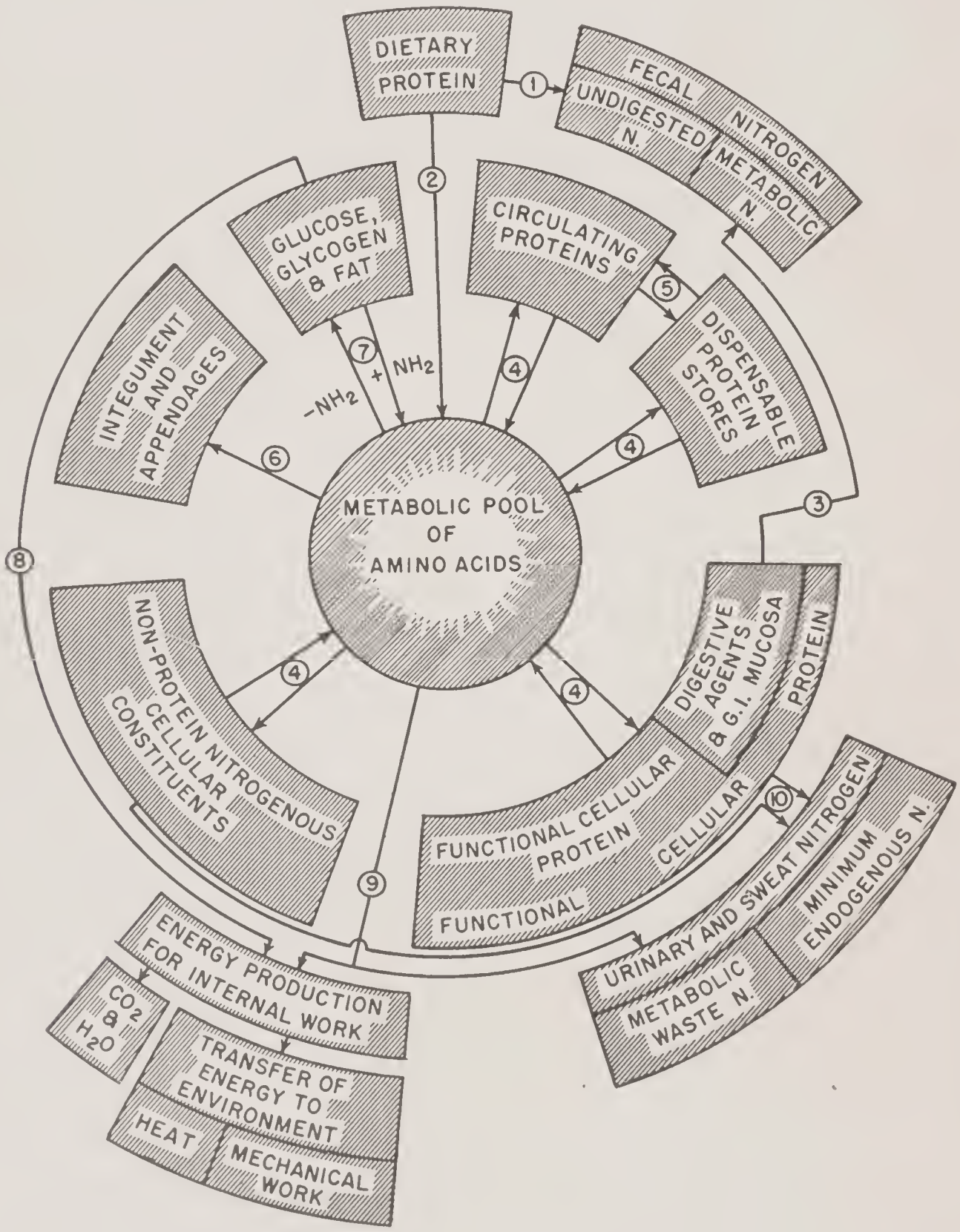

Fig. 1. A diagrammatic representation of protein metabolism in a sexually inactive animal. The significance of the numbers on the flow lines are explained in the text.

carbon. These studies revealed that protein synthesis was most active in the intestinal wall and in the pancreas.

"The intestinal wall secretes enzymes and mucous-proteins which are lost in enormous quantities (unlike other enzymes and proteins within the body). To compensate for this loss the intestinal wall may be more active in protein synthesis than other organs. If this explanation is correct, then the pancreas should also show a high turn-over rate; pancreatic juice contributes many enzymes needed for digestion. Actually Tarver and Schmidt found that the pancreas has the second highest specific activity among the organs of animals treated with isotopic methionine" (89).

(4) The reversible reactions occurring continuously on a protein-containing diet between the dietary amino acids, on the one hand, and the body proteins and nonprotein nitrogenous constituents, on the other hand. These are the reactions revealed by the isotope technic of Schoenheimer and his associates.

(5) These reactions between the circulating proteins of the blood, mainly the blood plasma proteins, and the dispensable protein stores in all tissues of the body, have been recently described by Whipple (47).

(6) The synthesis of the keratins of hair, wool, epidermis, nails, claws, hoofs, etc. The continued growth and regeneration of these protective proteins against environmental stress are sufficiently important to the body that, in case the food supply is inadequate to support them, the protoplasmic tissues of the body are raided to supply the amino acids, particularly methionine-cystine, needed, *via* the metabolic pool. In the adult animal, the protein requirements relate to the replacement of metabolic nitrogen lost in the feces (3), the endogenous losses in the urine (10), and the growth of the integument and its appendages (6). If the animal is of the "fur-hairiness" type according to the Friedenthal classification (31), like the albino rat, the amino acid requirements for keratin synthesis will dominate the total amino acid requirements.

(7) The storage of protein energy, after denitrogenation, as glycogen and fat. These reactions are reversible, if only to a limited extent, the amino group being provided by the metabolic pool.

(8) The oxidation of proteinogenous glycogen and fat to provide energy for internal work, later dissipated as heat, and for mechanical work.

(9) The direct oxidation of amino acids for energy production; also, some direct leakage of amino acids through kidney, sweat glands, and possibly epidermis in the insensible perspiration.

(10) The irreversible reactions involving functional cellular proteins, the indispensable proteins of Whipple (47), and the nonprotein nitro-

genous constituents of the cells, preeminently creatine, constituting the endogenous catabolism of Folin. The replacement of these losses and of the metabolic fecal losses, together with keratin synthesis (5) and other types of "adult growth" constitute the maintenance protein requirement of the adult animal in a nonstress environment. When the energy supply of the animal is inadequate and the deficiency cannot be currently supplied by body glycogen or fat, or under stress conditions involving hyperthyroid activity (70) and possibly other endocrine disturbances, the endogenous catabolism is accelerated and generally a creatinuria results.

VIII. Summary

From a study of the utilization by the growing rat, the adult rat and adult man of the nitrogen of a series of 6 proteins possessing limiting deficiencies of either lysine or methionine-cystine, it was shown that the biological values were higher for the adult rat than for the growing rat and for adult man when the dietary protein was deficient in lysine, and they were lower when the dietary protein was deficient in methionine-cystine.

The proteins of whole egg, prepared in the laboratory, appear to be slightly deficient in lysine for maximum utilization by the growing rat.

When the utilization of the nitrogen of the 6 proteins tested in adult nutrition are expressed as the amount of absorbed nitrogen per calorie of basal heat required for nitrogen equilibrium, the values are of the same order of magnitude for the rat and the human, but are definitely larger for the rat in the case of proteins deficient for growth in methionine-cystine, and definitely smaller in the case of proteins deficient for growth in lysine.

These relationships indicate that the cystine-methionine requirement is relatively more intense for the adult rat than for either the growing rat or for the adult human. On the other hand, the lysine requirement seems to be much less prominent among the amino acid requirements of the adult rat than among those of the growing rat or the adult human.

The reason for these relationships appears to be traceable to the relative prominence of keratin synthesis for the growth of the integument and its appendages (hair, wool, etc.). In the adult rat, in which protoplasmic growth is minimal but hair growth over the entire body continues, keratin synthesis, with its high requirement for cystine and its low requirement for lysine, histidine, and phenylalanine, dominates the total amino acid requirements.

This conception of the importance of keratin synthesis in determining the amino acid requirements of animals is fortified by evidence obtained

from studies of feather growth in molting hens and wool growth in sheep.

A corollary from this conception is that the total amino acid requirements of an animal of any age or species are determined by the proportions of the essential amino acids contained in the tissues being currently formed, or being currently catabolized.

In partial confirmation of this theory, striking similarities and high correlations are shown to exist between the proportions existing among the essential amino acids of the dominant tissues of the animal body, and the proportionate requirements for these amino acids as determined by feeding experiments with rats and chicks.

A method is proposed and illustrated for approximating the amino acid requirements for maintenance from the amounts of absorbed nitrogen per basal calorie required for nitrogen equilibrium in the form of a series of proteins of variable value in adult nutrition.

Finally, a theory of protein metabolism is proposed, and illustrated by diagram. This theory is based upon the Folin conception of two distinct types of protein catabolism, but includes later developments, especially those of Whipple and Schoenheimer and their associates, and the developments presented in this chapter, particularly the role of keratin synthesis.

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Methods of Measuring the Nutritive Value of Protein Hydrolysates and Amino Acid Mixtures. The Rat Repletion Method

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CONTENTS

I. Introduction 34
II. Protein Evaluation Methods—The Older Methods 35
1. Growth Method and Protein Efficiency Ratio 35
2. Nitrogen Balance Methods 36
a. Biological Value 36
b. Nitrogen Balance Index 37
c. Egg Replacement Method 38
3. Endogenous Nitrogen 39
4. Recent Experiences with the N-Balance Method 40
III. Assay Methods Based on Protein Regeneration 43
1. Regeneration of Labile Liver Cytoplasm 43
2. Maintenance of Protein-Depleted Rats 43
3. Rat-Repletion Methods 44
a. Assay of Dry Protein Hydrolysates 46
b. Assay of Liquid Protein Hydrolysates 47
4. Critique of Repletion Methods 51
a. Depletion—Length of Depletion Period and Type of Diet . . . 51
b. Methods of Feeding Nitrogen Supplements and Remainder of Diet 52
c. Effects of Different Feeding Levels of Nitrogen 53
IV. Methods of Chemical Scoring 54
1. Discussion of Methods 54
2. Limiting Effects of Amino Acid Imbalance 58
V. Specificity of Amino Acid Requirements 59
1. Essential Amino Acids 59
2. The Role of Nonessential Amino Acids and other Sources of Nitrogen 59
3. The Role of Arginine 62
4. The Role of Glutamic Acid 64
5. Ratio of Essential Amino Acid N to Nonspecific N 64
6. Experimental Approaches Used by Three Different Laboratories . . . 64
7. Significance of Recent Findings 66
VI. Racemic Amino Acid Mixtures 67
1. The Question of Inhibition by D-Amino Acids 67
2. Present Implications 70
VII. Summary 70
References 71

I. Introduction

The determination of the nutritional value of proteins is inherently less specific than, for instance, that of the vitamins. One deals from the assay standpoint with the evaluation of a single vitamin at a time. In the assay of protein value, conversely, one must be concerned with the qualitative and quantitative adequacy of at least 9 amino acids. There are also the problems of amino acid balance and utilization, and the relationship of calories, because amino acids can serve both as a calorie source and for building tissue, as needed.

In a recent resurgence of interest in protein nutrition, the classical methods of assay of protein value have been reexamined. This widespread renewal of research interest was timely with regard to advances in knowledge of nutrition in general, and with regard to the newer knowledge of vitamin and amino acid requirements in particular. Basal diets specifically deficient only in amino acids are now in general use.

Certain new methods of assay have been introduced and developed, and provide part of the subject matter of this discussion. Despite the fairly exact knowledge of the amino acid composition of proteins and the nutritive role of amino acids, it is inevitable that different assay methods show some differences in classifying proteins as to nutritive value. This is true because the different assay methods have differing parameters of measurement, and quite different endpoints. In this regard, attention is called to the collaborative study sponsored by the Bureau of Biological Research of Rutgers University in which 6 reference proteins are being evaluated and compared by many different methods of assay. This study may provide a fairly critical basis for comparison between certain of the methods themselves.

The need for rapid and precise methods of assay of protein value has received impetus in recent years through the increasing use of proteins and protein hydrolysates in medical practice. The Amino Acids Advisory Committee of the United States Pharmacopoeia has been particularly concerned with the nutritive evaluation of intravenous protein hydrolysates. In this regard, it is interesting to speculate on the eventual design of amino acid solutions for parenteral therapy. Following the discovery of threonine and the classification of the essential and the nonessential amino acids by Rose and his coworkers (1), there came the question of the nutritive role of the nonessential amino acids. Obviously the problem of devising a complete nutritive amino acid mixture would be more complicated by the requirement for each additional amino acid, over and above the 8 or 9 amino acids now recognized as being indispen-

sable. The data at hand suggest that simple, available nitrogen compounds have maximal sparing action for the essential amino acids in normal or protein-depleted rats. This advantageous situation may, of course, not always hold true in pathologic states. The experimental reports which throw light on this subject are discussed herein.

There is the further question in considering the use of synthetic amino acids of the physiological role and possible inhibitory effects of the unnatural D-amino acids. Practical methods of resolution of racemic amino acids are faced with as many imponderables as are the problems of synthesis. Because of this dual situation, protein hydrolysates are generally favored economically over synthetic, or even semi-synthetic, mixtures. Certain recent findings appear worthy of mention in any current attempt to place the unnatural amino acids in proper perspective in the unfolding picture of amino acid nutrition.

Finally, the establishment of the essential amino acid requirements of adult men (1) and of growing rats (2) at the University of Illinois, and of adult rats (3) and adult protein-depleted rats (4) at the University of Chicago provides a basis for relating rat requirements directly in terms of human requirements. Estimations of human amino acid requirements have also been made recently which appear well founded as a basis for correlation. Confidence is needed in the application of animal methods to the standardization of therapeutic amino acid preparations, and the exact knowledge of different species requirements has particular significance toward this end.

II. Protein Evaluation Methods—The Older Methods

1. Growth Method and Protein Efficiency Ratio

The way in which different proteins support growth in young rats has been the most general and widely used criterion of protein value. In 1919 Osborne, Mendel and Ferry (5) introduced the concept of "protein efficiency ratio" as a refinement of the simple growth method. The grams weight gain per gram protein intake were measured for several proteins and it was found that varying levels of protein in the diet gave different protein efficiency ratios. A rather definite level was found for each individual protein which produced the greatest gain per gram protein ingested. These levels for casein and lactalbumin were 12 per cent and 7.9 per cent respectively. In general, it has been found that the better the protein, the lower the level in the diet required to produce the highest protein efficiency ratio. This is a clear reflection of the importance of the proper nutritive balance of all of the amino acids

to produce optimum metabolic efficiency. In practice, however, it became fairly customary to determine protein efficiency ratios at a level of 10 per cent of dietary protein. Mitchell (6) criticized the method in 1944 on the basis of certain of the weaknesses which were recognized by its originators.

Barnes and his associates, working with mice, made a careful study of the protein efficiency method which led Bosshardt, Ydse, Ayres and Barnes (8) in 1946 to a new, improved and shortened technique. It may be mentioned again, however, that Block and Mitchell (7) have criticized these refinements in the method as being too cumbersome to be generally followed. The latter criticism may, of course, be voiced for the nitrogen balance methods as well, depending on one's experience and accommodations. Hegsted and Worcester (9) appear to have brought the most cogent criticism of the protein efficiency ratio method as ordinarily practiced. They have established that in a large series of experiments the protein efficiency ratios almost exactly parallel the rates of weight gain. Thus, in the ordinary comparison of proteins by the growth method, nothing may be gained by the calculation of protein efficiency ratios over and above the information obtained from direct comparison of the growth increments themselves, and the extra effort of measuring daily food intakes is avoided.

Nitrogen efficiency ratios have been calculated in this laboratory under the conditions of the rat-repletion method, wherein the rats are allotted a definite amount of amino acid nitrogen daily. In instances where rats do not take the complete allotment of nitrogen, the figure for nitrogen efficiency ratio provides a useful reflection of the net nutritive values of various preparations. In instances where animals consume the full allotment of nitrogen, comparison of weight increments alone is sufficient. It may be mentioned that the basis of feeding levels used in this laboratory is nitrogen, rather than protein. This seems appropriate in view of the considerable difference in nitrogen content of different proteins, and even between different samples of the same protein.

The growth method of assay obviously classifies proteins according to their adequacy to meet the needs for tissue synthesis, and is generally considered a critical index of protein value. The method is most critical when proteins are fed at a near-minimum level, *i.e.*, 1 to 1.5 per cent nitrogen added to a protein-free diet.

2. Nitrogen Balance Methods

a. Biological Value. The second of the older methods of protein assay is the method of determining biological value from the data on

nitrogen intake and excretion. The concept of "biological value" was first introduced by Thomas (10) in terms of per cent of digestible nitrogen from a test food which was retained by the adult human. Mitchell (11), on the other hand, applied the method to the growing rat, thus including the requirements for both growth and maintenance. Mitchell *et al.* (12) brought the method to an eventual high stage of precision as applied to the growing rat. The method involves paired feeding. In this regard, it is worthy of note that Hegsted and Haffenreffer (13) have recently presented evidence and a rationale in support of *ad libitum* feeding, particularly in regard to calorie intake. The many ramifications of such questions as this are beyond the scope of this paper. It may be argued, however, that the biologic value method presents fully as many imponderables and problems, and is no more precise than the growth or repletion methods of protein assay as now carried out with rats.

b. Nitrogen Balance Index. As shown by Allison *et al.,* (14, 14a) when adult dogs are highly standardized so that the so-called endogenous excretion is fairly constant, it is possible to plot absorbed nitrogen against nitrogen balance to obtain a straight line. The linearity holds, however, only in the region of negative balance and slight positive balance, indicating that reliable values can be obtained only at critical intake levels. The application of "nitrogen balance index" is essentially similar in practice to that of "biological value." Melnick and Cowgill (15) had previously pointed to a linear relationship between nitrogen balance and per cent protein calories in the diet. It should be recognized that these relationships do not in themselves add anything essentially new to the method of determining biologic value. Indeed if these relationships did not hold true for carefully executed experiments, it would be evidence that the method is unreliable.

The problems encountered in determining biological values or nitrogen balance indices in adult rats and dogs are also encountered, and possibly even to a greater and less controllable degree, in experiments with humans. For this reason, the clinical determination is fraught with much difficulty and uncertainty. Hoffman *et al.* (78) have applied the nitrogen balance index in human studies and have pointed to the following disadvantages: (1) the requirement of a long, tedious, difficult balance study; (2) the inherent error of the estimation of endogenous nitrogen excretion; and (3) the difficulty in drawing an accurate straight-line curve. The study, to have a reasonable degree of accuracy, must extend over a period of at least 3 weeks. The accuracy of the curve relating nitrogen balance to nitrogen intake depends primarily on the establishment of the endogenous nitrogen excretion. The latter is not

really a constant, and a figure of even relative constancy is not easy to determine. Nitrogen loss on nitrogen-free diet decreases rapidly at first and then gradually as body protein stores are depleted. Smith (79) in 1926 reported that endogenous nitrogen excretion had not become stabilized even after 24 days of a nitrogen-poor diet. Thus, the state of the patient with regard to protein need is always open to conjecture. The third consideration, the slope of the curve, depends upon the accuracy of a number of points. The variables may be so great as to produce a succession of points for which no line can be drawn. Too few points may falsely give a semblance of a straight line and be even more misleading.

An experiment under closely controlled conditions in this laboratory (22) in which dogs were maintained for 15 weeks on low levels of intravenous protein hydrolysate gave a fair graphic picture when all points were plotted. The nitrogen balance indices calculated from the slopes of the lines for the two dogs used in the experiment were 0.83 and 0.86, which are satisfyingly close. The fact may be stressed in defense of the method that the ability to construct a rectilinear curve from a series of nitrogen balance values provides valuable authentication of the over-all determination. Bricker, Mitchell and Kinsman (80), using essentially the same technique as that of Allison and his associates, studied natural food sources of protein with regard to minimum requirements in adult humans. They confirmed the parallel response to various levels of dietary protein intake even though individual test subjects were found to differ as to nitrogen balance requirement.

The weaknesses of the nitrogen balance method are for the most part greatly minimized by conducting the experiments over a fairly extended period so that internal compensation of protein deficiencies does not play a significant part. Despite this truism, it should be recognized that maintenance of nitrogen balance alone is not a guarantee of protein adequacy. The hypoproteinemic animal may come into bare nitrogen balance at low levels of nitrogen but continue to lose weight. The well-fed animal requires much more nitrogen from a given source to maintain equilibrium than does the depleted, or standardized, animal. The well animal remains in obvious health and buoyant spirits, and, in so doing, appears quite prodigal of food nitrogen. The depleted animal, on the other hand, has great avidity for nitrogen and this avidity is not ordinarily satisfied except by a prolonged period of high protein feeding. This situation is difficult to reconcile with the exact analytical requirements of the nitrogen balance methods as applied to the assessment of protein value.

c. Egg-Replacement Method. This method is essentially the deter-

mination of biological value as proposed by Thomas and by Mitchell, and has been carried out chiefly by Murlin and his coworkers in studies with adult humans at Rochester. Sumner, Pierce and Murlin (18) observed in 1938 that egg protein is of outstanding value for maintenance of N balance. Egg protein was found to have a biological value near 100; that is, the protein was almost completely absorbed, and there was no urinary loss of nitrogen over the normal loss on protein-free diet. This led to the concept of determining "biological value relative to egg." It also led to the practice of standardization of test subjects on maintenance levels of a high biologic value protein, rather than on nonprotein diet. Values for "endogenous nitrogen excretion" obtained when the subject is on a very low level of protein are certainly as reliable as values obtained when the subject is on nonprotein diet. Furthermore, protein-depletion is avoided by the first procedure. The most recent of a series of 6 papers from the University of Rochester group (19) is concerned with the determination in adult humans of the biological value of the 6 reference proteins distributed from Rutgers University.

3. Endogenous Nitrogen

It is generally agreed that Folin's theory of endogenous and exogenous protein metabolism is no longer tenable *in toto*. Borsook (17a) has recently reviewed the question of protein turnover and rate of amino acid utilization. As he points out, it has become increasingly clear that the rate of turnover of body protein in animals in N-balance is far greater than was envisaged by Folin's theory. The effects of endocrine stimulation of nitrogen excretion and retention are dynamic and may at any time serve to jeopardize the value of N-balance determinations.

Block and Mitchell (7) have defended the concept of endogenous nitrogen as applied in the calculation of biological value and have pointed to the independence of the conversion of creatin to creatinine by dietary amino acids as a case in point. Murlin (15a) has perhaps amply expressed the viewpoint of those who accept the concept of endogenous nitrogen as meaning "merely that a certain amount of nitrogen is excreted when no nitrogen is ingested." Berg (16) has also resolved the argument over the actuality of "endogenous nitrogen" and concludes as follows: "The change needed is one of viewpoint. What was once considered static is now proved only to have appeared so. The state which actually obtains is one of equilibrium, involving the summation of a complex assay of interdependent equilibria between opposing dynamic forces." Murlin, Szymanski and Nasset (17) have made the

interesting observation that the per cent of total N excreted as creatinine varies for different proteins and that close correlation can be drawn between this percentage figure and the biologic value of the protein fed. Creatinine nitrogen is remarkably constant on a creatine and creatinine-free diet and this constancy adds workable credence to the concept of "endogenous nitrogen" excretion.

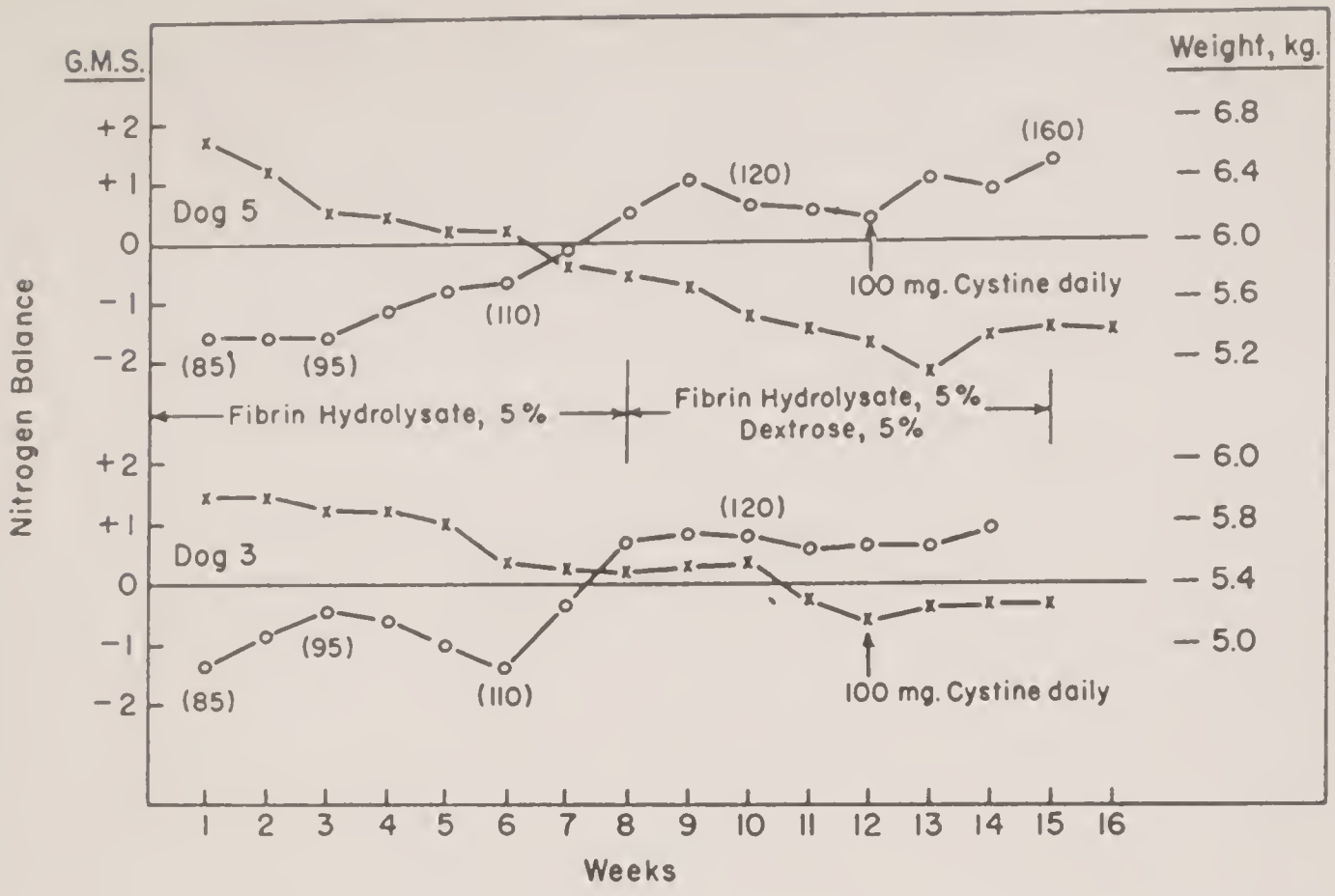
4. Recent Experiences with the N-Balance Method

Early in the work on intravenous protein hydrolysates in this laboratory, Risser, Schenck and Frost (21) concluded on the basis of short-term nitrogen balance studies in dogs that there was no difference in the biological value of partial acid hydrolysates of both casein and fibrin over a wide range of hydrolysis, extending to complete hydrolysis. Later work, in which dogs were maintained over very long periods of time (22), and in which severely protein-depleted dogs were used (23), led to somewhat different conclusions and demonstrated the need for considerably more heroic measures than the simple 4 or 5 day nitrogen balance method to provide a final answer. Results of these types of study are shown in Figs. 1 and 2.

Recently Allison, Chow, White and Roth (24) reported that wide variations in the degree of enzymatic hydrolysis of casein did not significantly alter the nitrogen balance index as determined in 4-day periods. The dogs were consistently in negative balance at an intake of 120 mg. N per kilo per day. Lactalbumin was fed during the 3-day periods before and after injection. The findings are comparable to the early findings in this laboratory, except that our dogs were essentially in balance. Our subsequent findings, however, would cast much doubt on the real significance of such short-term experiments.

We may also make general note of the experience of the 6 laboratories which collaborated in the U. S. P. study of the nitrogen balance method for the standardization of intravenous protein hydrolysates. The study was carried out under the chairmanship of J. B. Allison and the author, and the method used was essentially that of Allison, Seeley and Ferguson (25). The results of the general study showed great variation both within and between laboratories although some classification of the different products could be made in a broad way. The study clearly demonstrated the need for careful standardization of the test animals, but failed to provide a highly critical and practicable method of standardization. A primary criticism of the method was the inadequacy of the 4-day period to allow a good resolution of differences.

A further point of interest is found in the report of Silber and Porter



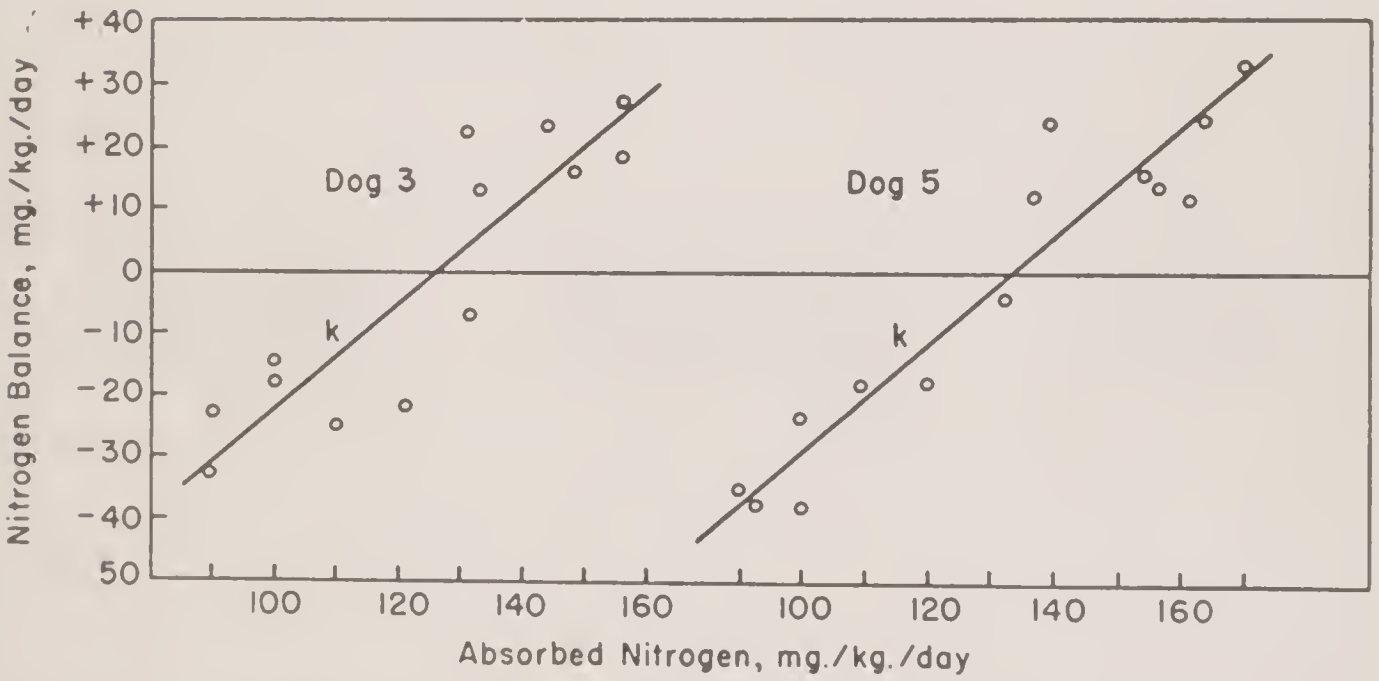


Fig. 1. The upper figure shows weight and nitrogen balance curves for 2 dogs which received partial acid hydrolysate of fibrin intravenously as the sole source of amino acid nitrogen. Injections were made daily for 14 weeks. The rate of injection was 1 mg. N per kilo body weight per minute. The level of nitrogen injected was varied and is shown in parentheses in mg. N per kilo per day. Despite the fact that they came into balance at the 110 mg. N level, it will be noted the dogs continued to lose weight.

The lower figure is a plot of nitrogen balance against absorbed (injected) nitrogen for the 2 dogs, above, for the 14-week period. The slope of the line, k, according to Allison's formula (Nitrogen balance $= k$ (absorbed nitrogen minus endogenous nitrogen)) represents that fraction of the injected nitrogen retained in the body, the biological value, or nitrogen balance index. The value for endogenous nitrogen was taken as the average excretion on a low-protein standard diet. This average value is in the range 90-100 mg. N per kilo per day.

(26) who have described the results of their own laboratory in the above collaborative study. These authors reported a zero biologic value for one preparation, when injected, as opposed to good utilization when the product was given orally. The general conclusion was that there was a significant loss of peptides following injection of all partial hydrolysates and that certain products were markedly less well utilized by injection

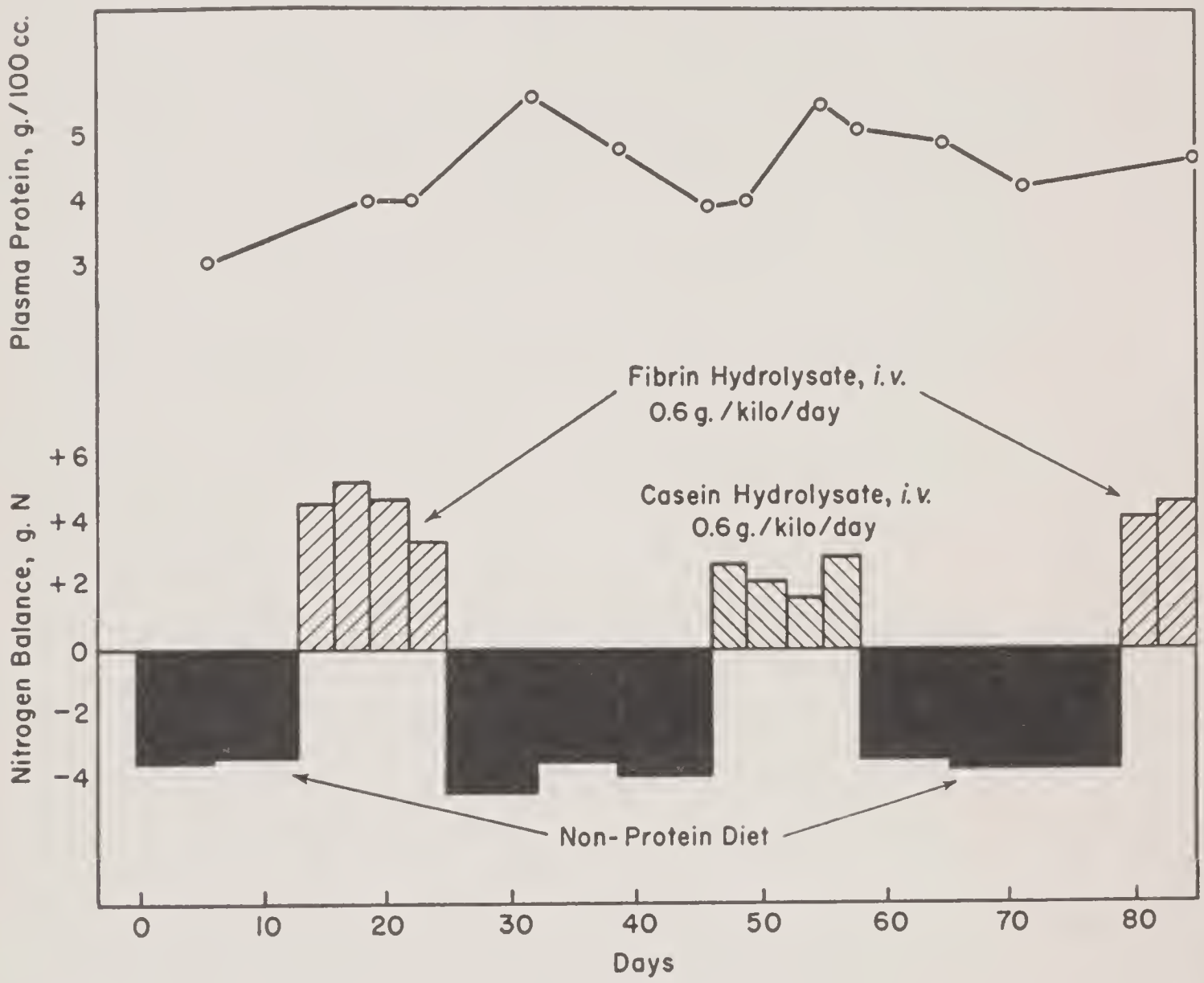


FIG. 2. Graphic presentation of the sequence of repeated protein deprivation on nonprotein diet and regeneration on massive intravenous therapy. The graph corresponds to the data mentioned in the text (23), and shows the method of comparing the ability of two different hydrolysates to correct severe experimentally induced hypoproteinemia.

than by oral dosing. These results are quite contrary to those of Allison *et al.* (25). In the author's opinion, it is rather fruitless to draw final conclusions on such points of difference without recourse to more rigorous criteria, such as long-term balance studies at minimum nitrogen levels and the correction of induced hypoproteinemia. As shown by Weech (27), the rate of plasma protein regeneration is a useful criterion of protein value in the latter type study.

In the experience in this laboratory, the short-term method of deter-

mining biological value, or nitrogen balance index, in adult dogs is not as useful for resolving differences in protein and protein hydrolysate values as are the rat-repletion or rat-growth assay methods. The large quantitative differences between requirements for growth and maintenance may account in part for the differences in discerning power of the two types of assay. Hegsted, Hay and Stare (20) have estimated that the tryptophan requirement per kilo body weight for growth of rats versus nitrogen balance in dogs are 94 and 1.6 mg., respectively, and for isoleucine 880 and 15 mg., respectively. In the normal adult animal there is clearly a reserve, however small, of protein which makes for equilibrium. In the depleted or growing animal, conversely, there is no reserve and all of the driving mechanisms of the body are in the direction of protein storage. The quantitative day-to-day requirements for equilibrium are not nearly so critical as are those for growth or repletion.

III. Assay Methods Based on Protein Regeneration

1. Regeneration of Labile Liver Cytoplasm

Addis and his collaborators in 1940 (28) examined the relationship between dietary protein intake and protein content of the liver and found it to be a fair measure of protein value. Kosterlitz and Campbell (29) developed rapid methods based on the estimation of labile liver cytoplasm following brief fasting in rats, and Harrison and Long (31) confirmed the general method. The methods are based on the fact that adult rats fed protein-free diet lost practically all labile liver cytoplasm in a few days and that the rate of regeneration is a fair measure of the nutritive value of a test protein. The method has been examined statistically and reduced to as simple a form as possible by Campbell and Kosterlitz (30). The method even in simplest form, however, requires several determinations on the excised livers of highly standardized animals, and, therefore, appears somewhat formidable as compared with methods based directly on body weight gain under equally closely defined conditions.

2. Maintenance of Protein-Depleted Rats

Tomarelli and Bernhart (32) have described a method which measures the amount of nitrogen from any protein which is required to maintain constant weight in protein-depleted rats. The method calls for a one-week depletion of adult rats on nonprotein diet. Separate groups are then fed varying levels of the test samples and standard protein and the effects on weight are observed for one week. The weight

change plotted against N intake provides an index to maintenance requirement, which is evaluated by comparison with the casein standard. Neither of the above methods have been widely studied. Nor have they been standardized in terms of the requirement for each of the individual essential amino acids. Both methods appear, nevertheless, to offer certain advantages in special situations, which may recommend them for more thorough investigation.

3. Rat-Repletion Methods

Cannon, Humphreys, Wissler and Frazier (33) devised a fairly rapid method of protein assay as a tool in their studies of the relationship of protein metabolism to antibody production and resistance to infection. The latter relationships and the pathologic consequences of protein and amino acid deficiencies have been reviewed by Cannon (4, 34). As pointed out by Cannon, the principle utilized is similar to that used often in biology, *viz.*, the production of a biological deficit in order to measure the replacement value of a material to be tested. The method can demonstrate variations in protein quality in one to two weeks. Adult protein-depleted rats show great avidity for protein supplements, and typify the clinical conditions wherein the use of protein and balanced amino acid feeding is most clearly indicated. According to Cannon (34) also, the method has shown excellent agreement with the rat-growth assay in unpublished experiments for the Quartermaster Corps.

In the early work (33) on the rat-repletion method, a close parallelism was noted in the capacity of proteins to promote both weight recovery and plasma protein regeneration. The specific effects of the essential amino acids for the two functions were exactly shown in two concurrent papers by Frazier, Wissler, Steffee, Woolridge and Cannon (35) and by Benditt, Humphreys, Straube, Wissler and Steffee (36). In the first paper, the requirement in the adult rat for each of the 9 amino acids, previously shown by Rose to be essential to the growing rat, was established. The omission of any one of the 9 essential amino acids led to prompt loss in appetite and weight. The promptness and clear-cut effects on appetite and weight gain upon restoration of the individual missing amino acids likewise leave no doubt as to their essentiality and profound physiologic effect. The findings clearly demonstrated also the surprising fact that food acceptance by protein-depleted rats may be determined by the presence or absence of only a few mg. of a particular essential amino acid. Cannon (4) has tabulated the approximate daily requirement in milligrams for rapid weight recovery for each of the 9 essential amino acids and has shown that the ratio of these requirements is very

TABLE I

Comparison of Rat Requirements for the Essential Amino Acids for Growth, Repletion and Maintenance

	Repletion requirement (4, 75)		Requirements for maintenance of weight (38)		Growth requirement (39)	
	mg./rat day [a]	*Ratio*	*mg./100cm.²/day*	*Ratio*	*% of diet*	*Ratio*
Tryptophan	14.5	1	2.2	1	0.2	1
Phenylalanine	45	3.1	6.0	2.7	0.7	3.5
Leucine	72.5	5	8.0	3.6	0.8	4
Isoleucine	61	4.2	13.7	6.2	0.5	2.5
Methionine	39	2.7	7.3	3.3	0.6	3
Threonine	43	3.0	5.3	2.4	0.5	2.5
Lysine	58	4.0	4.5	2.0	1.0	5
Histidine	21.5	1.4	2.2	1.0	0.4	2
Valine	51	3.5	10.0	4.5	0.7	3.5

[a] These values, which are somewhat different from those first reported (4), are the preferred values and are used here through the courtesy of Dr. Paul Cannon and his collaborators. (75)

close to the ratio in which these amino acids occur in whole egg protein.

The requirements for maintenance and for repletion in the adult rat have been shown to be qualitatively similar by Wissler *et al.* (37). The quantitative requirements for maintenance of N balance and of weight have been reported by Frazier *et al.* (38). The ratios of these requirements are of interest and differ only in a few instances, one from the other, as shown in Table I. Rose's (39) minimum requirements for rat growth are also shown. The difference in ratios of requirements for the individual amino acids is not large enough in any of the 3 situations to be considered significant, except possibly in the case of lysine, in which the ratio of requirement for growth and repletion is higher than for maintenance. These data provide a better foundation for the comparison of methods based on growth, repletion and maintenance than has obtained heretofore.

a. Assay of Dry Protein Hydrolysates. The assay of various protein hydrolysates by the rat-repletion method has been reported by Wissler *et al.* (40). The following points were listed by the authors as advantages of the adult protein-depleted rat as a test animal to measure the potential value of protein hydrolysates:

(1) Its qualitative essential amino acid requirements parallel closely those of man.

(2) Such animals simulate conditions of protein depletion seen in many sick patients.

(3) The small size of the rat and its ready consumption of synthetic rations make the assay relatively simple and inexpensive.

(4) Protein depletion provides an increased stimulus for the fabrication of tissue and blood proteins. Consequently, in a relatively short experimental period, one can measure much larger increases in protein than those found in physiologic growth.

(5) It is possible to measure refilling of several protein compartments simultaneously. The authors point out further that weight recovery alone is sufficient as a measure of protein value, as this parallels the regeneration of plasma protein, hemolysin, hemoglobin, liver protein and total carcass protein.

The amino acid requirements of rats for growth, maintenance and repletion may now be compared with maintenance requirements for the adult human. The ratios of requirements of Table I may be compared with the minimum requirements for maintenance of N balance in adult humans, recently reported by Rose (1). Comparison may also be made with the calculated human maintenance requirements of Harte and Travers (41) and of others. Albanese (42) has further estimated the amino acid needs of infants and of children. The latter values obviously

provide a less secure basis for comparison than do the experimentally determined values. An objective should be to determine as exactly as possible the amino acid needs for growth in children and for optimum rate of protein regeneration in adult protein-depleted humans; however, such a program is beset with great difficulties and will certainly not be accomplished in the near future.

As a point of interest in Table II, we have compared the ratios of the rat-repletion requirements of Cannon, and the minima for N-balance in humans established by Rose and calculated by Harte and Travers. It is important to point out certain of the pertinent differences in the way in which the respective minima were established, as these differences have a bearing on certain of the requirements. For instance, the human minima (1) were established in absence of cystine and tyrosine, so that the methionine and phenylalanine requirements are absolute. The rat-repletion requirements were established, on the other hand, with a 16-amino acid mixture (35) containing small amounts of cystine and tyrosine, so that the methionine and phenylalanine requirements are not absolute. In each case it would appear that there was sufficient nitrogen other than essential amino acid N to spare the latter fully for tissue synthesis. Thus, the relative ratios of requirements for methionine and phenylalanine as determined for the human would be expected to be somewhat greater than that for the rat, as is indeed the case.

Comparing the ratios of requirements in Tables I and II, one has little to choose in selecting a method which is most representative of human needs. In general, one would say that methods based on growth, maintenance or repletion would be of equal value, and that the choice of method may rest with the inherent and desired accuracy of the methods themselves.

b. Assay of Liquid Protein Hydrolysates. The assay of liquid protein hydrolysates presents a variety of problems, as follows: (1) hydrolysates containing dextrose cannot be conveniently dried without loss of nutritive value; (2) dried hydrolysates are hygroscopic and, therefore, difficult to blend into dry rations; (3) weanling rats cannot, or do not, ingest sufficient volumes of dilute hydrolysate solutions to meet the needs for maximum growth.

Mueller (43) in 1945 reported the assay of liquid protein hydrolysates fed in dry or liquid form to weanling rats. Although the method was not eminently successful, it provided the first working basis for a rat assay. Frost and Sandy (44, 45) in 1948 adapted the "rat-repletion method" to the assay of liquid protein hydrolysates in protein-depleted rats. The method had a marked advantage over the growth method previously reported by Mueller in that adult protein-depleted rats are

TABLE II

Ratio of Minimum Daily Requirements of the Indispensable Amino Acids as Determined by Three Methods: Rat Repletion (4), Nitrogen Balance Studies in Adult Men (1), and Calculation from Minimum Protein Requirements for Maintenance in Adult Humans (41)

	Rat repletion requirement (4, 75)		Requirement for N-balance in adult men (1)		Calculated requirement for N balance in adult humans (41)	
	Mg./rat day	*Ratio*	*G./day*	*Ratio*	*G./day*	*Ratio*
Tryptophan	14.5	1	.25	1	0.4	1
Phenylalanine	4.5	3.1	1.1	4.4	1.4	3.5
Leucine	72.5	5.0	1.1	4.4	1.7	4.2
Isoleucine	61	4.2	0.7	2.8	1.2	3.0
Methionine	39	2.7	1.1	4.4	0.5	1.3
Threonine	43	3.0	0.5	2.0	1.0	2.5
Lysine	58	4.0	0.8	3.2	0.8	2.0
Histidine	21	1.4	—	—	0.5	1.3
Valine	51	3.5	0.8	3.2	1.1	2.8

able to consume very large volumes of liquid and show a much more uniform appetite for liquid protein hydrolysates than do young rats.

The question of liquid balance and liquid tolerance is an interesting one in itself with regard to the way in which animals of various ages can and will take liquid nutrients. Robinson and Adolph (46) have established that a deficit of water relative to other body components provides a signal which initiates drinking in ordinary life. The amount of water drunk to correct the immediate deficit is ordinarily about 0.5 per cent of body weight. Under extraordinary urges to drink in order to obtain nutrients, however, adult rats may drink more than their own

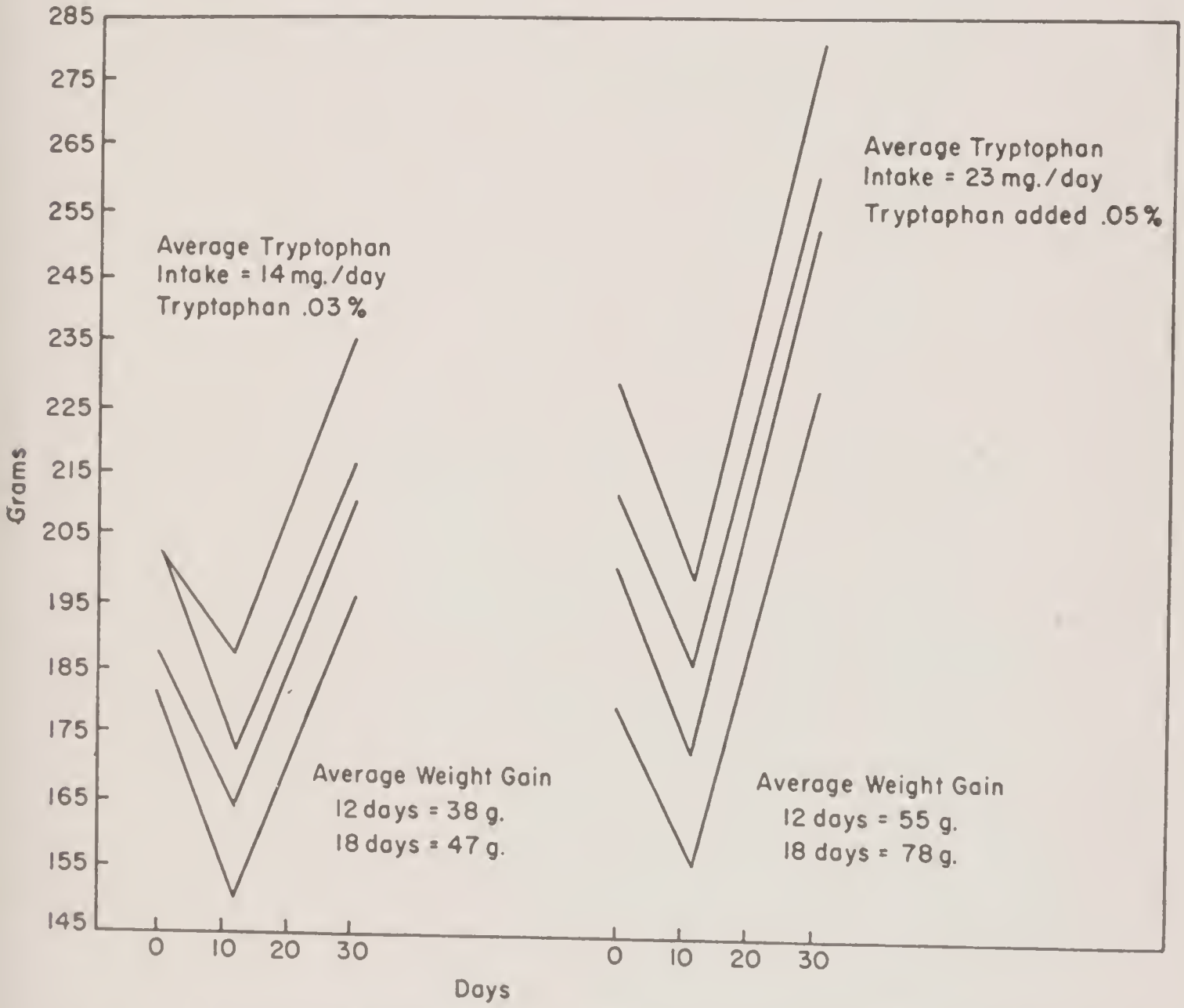


Fig. 3. *Ad libitum* feeding of a 5 per cent fibrin hydrolysate containing two different levels of tryptophan. Under these conditions, about 18 mg. tryptophan per rat day suffices for maximal weight gain. Cannon's minima, which are the levels required to produce a good rate of weight gain under a given set of conditions, are generally expected to be somewhat lower than the levels which may give a maximum response under other sets of conditions. Orientation of the animals to liquid feeding (drinking trial) was not carried out in this early study, although it has become customary in all of the more recent work. The figure is used principally to illustrate the effect of a partially limiting deficiency of a single amino acid.

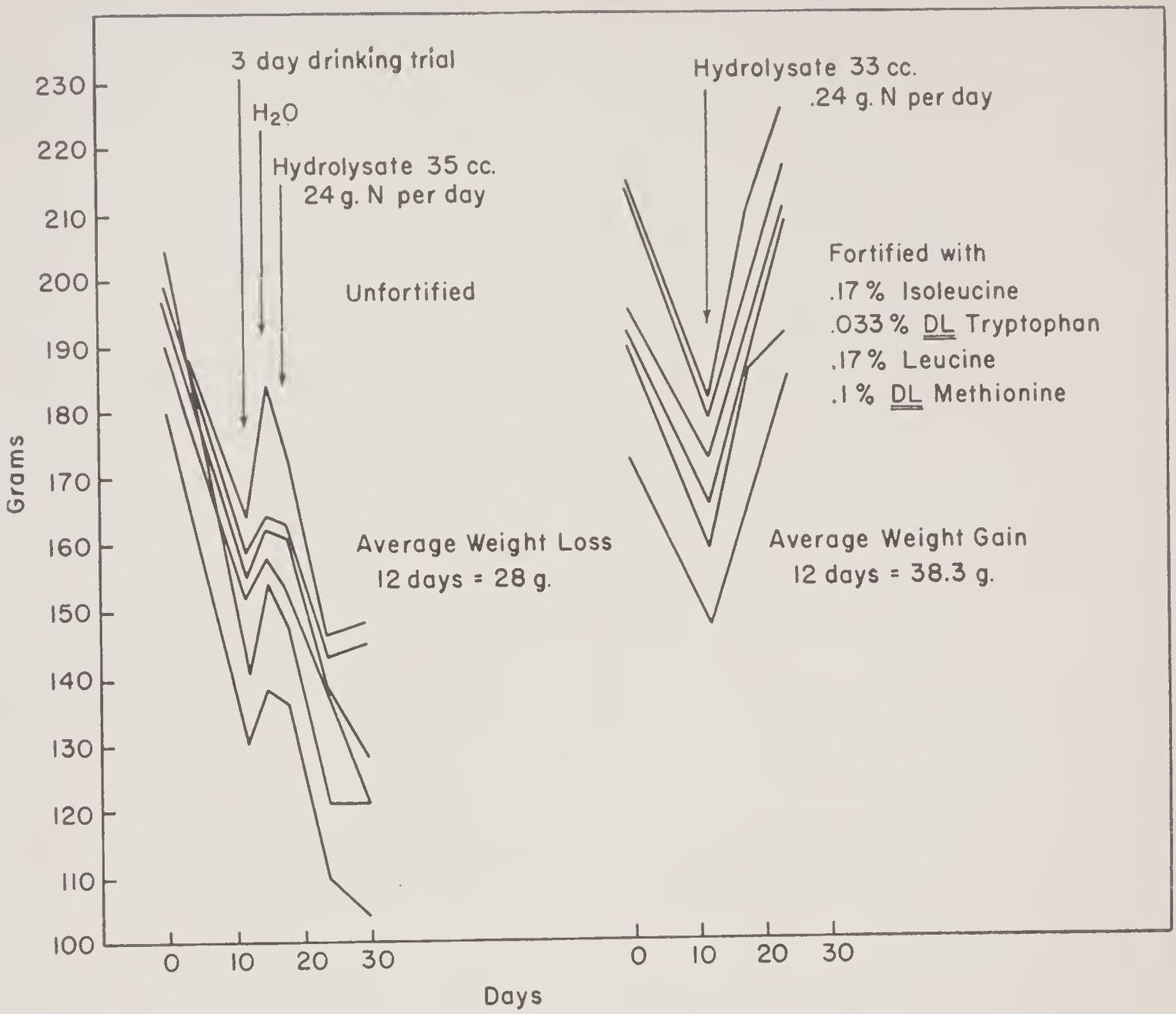


Fig. 4. Response of protein-depleted adult rats to a hog blood hydrolysate prior to and after fortification with certain amino acids known to be present in limiting amounts. The same gronp of rats was used throughout. Orientation of the animals to a large volume of liquid nutriment was carried out with a 5 per cent hydrolysate of high nutritive value. The rats all took 40 cc. per day of this preparation. They were then redepleted for 3 days on nonprotein diet and water, at which time the unfortified hydrolysate was introduced in place of water. Acceptance of the unfortified hydrolysate was very poor, amounting to only a few cc. per day. Following 12 days on the unfortified hydrolysate, the rats were repleted on stock diet. They were then again depleted before assay of the fortified hydrolysate. The full allotment of the latter was consumed each day.

body weights of water every 24 hours. Adolph (47) has reported that adult rats maintained weight on milk diluted to as low as 2.6 per cent solids, but only by drinking very large volumes sufficient to meet caloric needs. McCance and Wilkinson (48) have made the further pertinent observation that very young animals are less capable of handling large volumes of liquid than adult animals. These combined observations suggest that the adult protein-depleted rat is well adapted to the assay in question; whereas weanling rats are not as well adapted. Two studies,

not previously reported, of the rat repletion method with liquid hydrolysates, are shown in Figs. 3 and 4.

4. Critique of Repletion Methods

a. Depletion—Length of Depletion Period and Type of Diet. The method of assay of liquid protein hydrolysates (44, 45) differs in certain particulars from the method as originally developed and practiced by Cannon and his collaborators (33–38, 49). One of these differences is the time and conditions of depletion. The latter work was first carried out (33, 49) with a protein-low diet (3E) based on natural materials, including carrots, brewer's yeast and liver concentrate. In more recent studies (35, 36) by the University of Chicago group, adult rats were depleted for approximately 3 months on the protein-low diet (3E) and then transferred to protein-free diet (4E) during the repletion periods. In very recent studies (50, 51), the protein-free diet has been used for the depletion and repletion periods. In the first instance (35), male rats (220–244 g.) were depleted on ration 3E about 3 months. Animals were then chosen for repletion on the basis of uniformity in initial weights, percentages of weight loss (25–33 per cent), and concentration of serum proteins and hemoglobin. In these studies (50, 51), adult male rats weighing initially 288 to 350 g. were depleted of 15 to 25 per cent of their body weight in a period of 5 weeks on the protein-free diet.

Experience in this laboratory (44) was that male rats (140–220 g.) lost 21 to 28 per cent of body weight in 12 days on protein-free diet. The original weights of the rats in this range did not appear to have any marked influence on the per cent weight loss during depletion or the magnitude of the repletion response. The 12-day depletion period appeared entirely adequate to elicit a uniform and maximum repletion response. More recently a method has been developed (52) for routine control purposes with an improved basal protein-free diet wherein rats are used repeatedly with intermittent 7-day depletion periods between assays.

Optimal conditions of protein depletion for the purpose in question have as yet not been determined by direct experiment. One may assume that the object would be to thoroughly deplete the test animals of reserve protein without causing serious damage and without depleting to the point where edematous changes would mask true tissue weight loss. The following reports from the literature appear to have a fairly direct bearing on this question.

Addis, Poo, and Lew (54) in 1936 showed that the liver is the primary organ to suffer a serious loss of protein, 40 per cent being lost in a week's

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fasting. Kosterlitz (53) states that rats fed protein-free diet lose practically all labile liver cytoplasm in 4 days. Liver losses become much less rapid after the first few days, and thenceforth, according to Campbell and Kosterlitz (55), follow an exponential curve as protein starvation proceeds. Thus, the methods (29–31) based directly on regeneration of liver protein are of particularly short duration, allowing for only 2 to 7 days fasting or depletion on protein-free diet.

The recent complete study of Wang, Hegsted, Lapi, Zamcheck and Black (56) is perhaps most pertinent to the question at hand. It is apparent from these studies that the major changes in the liver N occur within the first 12 days of protein depletion. From this time on, subcutaneous edema appeared in a few of the animals, although the tendency toward edema was only slight. After about the 25th day of depletion, the liver returns toward normal, both in chemical and cytologic character. The authors interpret the latter phenomena as representing an attempt within the animal body to conserve liver tissue after the first primary loss of more or less dispensable material. One may judge from these data that the depletion period on protein-free diet would certainly not need to be more than 25 days, and that a period of 12 days may actually serve as well to accomplish the desired protein depletion.

b. Methods of Feeding Nitrogen Supplements and Remainder of Diet. A second question in the mechanics of the rat-repletion method is the manner of feeding the protein supplement. The procedure of the Chicago group (33–38, 50, 51) is as follows: the ration is so constructed that 1.35 g. of protein is obtained in 15 g. of ration, which supplies 48 calories. Consumption of the total allotment is reported to be complete for the better protein supplements, but is quite incomplete when an amino acid imbalance is presented (40). The avidity for the ration is, indeed, a fair measure of the value of the amino acid mixture which is incorporated in the diet.

The protein supplement may be fed separate from the remainder of the diet as reported by Frost and Sandy (52). This practice was an outgrowth of the separate feeding of liquid protein hydrolysate, and was found to work equally well for dry proteins. The protein supplements are weighed daily into cups, which are then attached to the side walls of the cages in such a way that the adult rats have easy access to them, but cannot upset them. Daily protein feedings on the order of .12, .2, .24 and .36 g. N per day have been used. The level of .12 g. N per day is highly critical, representing only about 1 per cent of the total diet consumed.

The avidity for protein in general is very great, and after a brief learning period, the protein supplements are immediately and regularly

consumed. The nonprotein diet is fed *ad libitum,* as it is felt the appetite for the remainder of the diet should be in no way limited but should reflect the full effect of the supplement on the capacity for recovery. It is very clear that protein-depleted rats will not take unbalanced amino acid mixtures either added to the diet (35), or fed separately from other parts of the diet (44). In this type of diet, then, in which all other components are presumed to be adequate, the most important appetite-conditioning factor is the quality of the protein component. For this reason it appears desirable to limit the feeding of the protein component at some desired critical level, and to allow *ad libitum* intake of the bulk of the diet.

Hegsted and Haffenreffer (13) have provided experimental evidence that nutritional deficiency automatically limits appetite, presumably by some mechanism related to total metabolism, which can or cannot proceed at maximum rate, depending on the balance and supply of all nutritive factors. One argument against paired-feeding of the entire diets is that no pertinent information is gained over and above what is learned from experiments wherein *ad libitum* feeding, particularly of calories, is practiced.

Ad libitum feeding of both the protein and nonprotein components of the diet in the rat-repletion assay was practiced in a few beginning experiments by Frost and Sandy (44) This type of experiment is applicable in assessing the general nutritive value of a protein, and particularly, the practical feeding value of a mixture of proteins. The procedure of Cannon and his coworkers, as described above, supplies an abundance of calories. The level of protein added to the basal diet is relatively low, only 9 per cent; however, an intake of 15 g. daily would supply 1.35 g. of protein, equivalent to about .2 g. of nitrogen. The latter level is at least twice the minimum required for a half-maximum rate of regeneration. One may judge that, in practice, this method allows essentially *ad libitum* consumption of calories, but a controlled feeding of the protein component. The point at question is whether or not there is advantage in limiting the caloric intake at all by tying it to the protein intake.

c. Effects of Different Feeding Levels of Nitrogen. Frost and Sandy (45) have studied the response to various controlled levels of nitrogen. The dose response curve for a fibrin hydrolysate was linear in the range .12–.3 g. N per rat day. Levels of .12 and .24 g. N per rat day were used (52) in the assay of the Rutgers reference proteins, and of beef blood fibrin and lactalbumin. Table III shows the rat-repletion response to these proteins at the two feeding levels, together with the per cent of requirement supplied by the most limiting amino acid in each protein.

The fact that, in each case, at least one of the essential amino acids is supplied at a level below the maximum is sufficient for the failure of any of these proteins to give a maximum response at this very low feeding level. It is obvious, also, that at the .24 g. N level, such proteins as egg albumin and whole egg will supply more than 100 per cent of the requirement, but wheat gluten and peanut flour still fall far short. There is evidence that the methionine of casein is not well utilized (45) so that the apparent superiority of casein over beef muscle appears as an artefact. The differences in proteins fed at levels which supply more than the full requirements of all amino acids for maximum performance may be accounted for on the basis of amino acid balance, a subject discussed later in this paper.

IV. Methods of Chemical Scoring

1. Discussion of Methods

Evaluation of the nutritive value of a protein from knowledge of its amino acid composition still leaves much to be desired. Nevertheless, it provides a real challenge and is capable of disclosing many useful correlations. It is particularly useful when considering the supplementary effects of mixtures of proteins, or protein derivatives. The early work of Osborne and Mendel (57) established the great improvement in the nutritive value of gliadin by the addition of lysine, and of zein by the addition of both lysine and tryptophan. More recent findings have established many other limiting deficiencies in specific proteins, such as the deficiencies of isoleucine and methionine in hemoglobin, or isoleucine and tryptophan in blood albumin, and of methionine in peanut and soybean.

Mitchell and Block (7, 58) have tabulated the essential amino acid content of many of the common food proteins and have compared them with whole egg protein as a standard of excellence. According to their formulation, the biological value of a protein (y) may be roughly estimated from its maximum percentage deficit in an essential amino acid (x) by the equation: $y = 102 - 0.634x$. As pointed out by the authors, although correlation between the experimental and derived values are fairly good in general, there are instances in which agreement fails. For instance, the nutritive value of cereal proteins may be greatly impaired by heat, although no change in the content of essential amino acids is apparent by analysis.

There are many other factors which operate against the clear application of the above method, particularly for comparisons between proteins

of intermediate or high nutritive value: (1) The composition of whole egg protein cannot be assumed to be in the exact ratio of minimum requirements for each amino acid; (2) analytical values for each of the essential amino acids are not irrefutable; and (3) in a few instances, whole egg protein has appeared somewhat inferior to certain other proteins.

One may also compare the composition of proteins with the established ratios of requirements, as is done to some extent in Table III. The ratio of the essential amino acids of whole egg (58) has been calculated as a matter of interest and is as follows: tryptophan 1, phenylalanine 4.2, leucine 6.1, isoleucine 5.3, methionine 3.7, threonine 3.3, lysine 4.8, histidine 1.4, and valine 4.9. The correspondence of the above ratio to the ratios of requirements of rats and humans (Tables I and II) is probably no better nor worse than the correspondence between any of the other sets of ratios.

There is rather general agreement following the classic work of Rose that the essential amino acid composition of a protein provides the master key to protein quality. It is clear also that proteins of high nutritive value contain all of the essential amino acids in good amount and in ratios not greatly different one from the other. Analytical data (52) on the Rutgers reference proteins showed that the essential amino acids accounted for about 60 per cent of the total weight of the better proteins, but only about 40 per cent of the poorer proteins. Both bioassay methods and methods of chemical scoring were easily capable of separating the better from the poorer proteins in this series, as shown in Table III.

On the other hand, the problem of differentiating the better proteins, one from the other, on the basis of their essential amino acid composition (52) as related to requirement is not clearcut as seen in Table IV. Although peanut flour and wheat gluten display easily discernible limiting deficiencies, this is not equally true for defatted whole egg, egg albumin, casein, and defatted beef muscle. The deficiency in casein is undoubtedly that of the sulfur amino acids, methionine plus cystine. Egg albumin appears to have slightly better composition to meet the needs of the protein-depleted rat than does whole egg, and this is borne out by the experimental results of Table III. Egg albumin is particularly rich in the sulfur amino acids, followed by whole egg, beef muscle and casein.

In any event, none of the proteins appears to have a composition which closely matches the ratio of rat-repletion requirements. Incidentally, also, the ratio of essential amino acids outlined for whole egg protein by Mitchell and Block (58) varies considerably from that determined for defatted whole egg in this laboratory (52). This observation

TABLE III

Rat-Repletion Responses to Proteins at Two N Levels. Limiting Amino Acid Deficit Calculated from Essential Amino Acid Content

Protein supplement	Repletion response				Limiting amino acid	% of requirement supplied [a]
	at .12 g. N/day, g.		*at .24 g. N/day, g.*			
Egg albumin	38	±3.5	80	±4.9	Isoleucine	70
Whole egg (defatted)	33	±1.4	66	±3.9	Isoleucine	70
Beef muscle (defatted)	31	±1.4	46	±3.5	Methionine Isoleucine	44 47
Casein	31	±2.9	45	±3.7	Methionine Tryptophan	64 69
Peanut flour	8	±6.3	32	±2.7	Methionine	13
Wheat gluten	9	±2.3	19	±2.4	Lysine	20

[a] The milligram quantities of each of the essential amino acids supplied in an amount of protein containing .12 g. N was calculated from the amino acid composition data (52) for each protein. In each case, as shown, at least one of the essential amino acids was present at suboptimal level as compared with Cannon's minima (4).

TABLE IV

Profile of Rutgers Reference Proteins as Compared with the Requirement for Each Amino Acid for a Rapid Rate of Repletion

Essential amino acid	Repletion minima [a] per 150 g. rat/day, mg.	Per cent of requirement supplied by each protein, at level of 120 mg. N per rat day					
		Egg albumin, %	Casein, %	Defatted whole egg, %	Peanut flour, %	Wheat gluten, %	Defatted beef muscle, %
Tryptophan	14.5	97	69	97	77	69	83
Phenylalanine	45	111	96	98	71	82	64
Leucine	72.5	97	101	94	58	68	77
Isoleucine	61	70	70	70	43	52	47
Methionine	39	79	64	67	13	26	44
Threonine	43	84	79	88	42	44	79
Lysine	58	77	91	74	32	20	103
Histidine	21.5	93	116	75	73	71	126
Valine	51	114	108	116	53	55	71

The values underlined indicate the probable limiting amino acids and the severity of the limitations—the limitations in methionine cannot be interpreted clearly because of the complemental effect of cystine, which is present in varying amounts in the different proteins (see text).

[a] Values slightly revised from former published values (4); communication from L. E. Frazier and Dr. P. R. Cannon (75) who kindly made the values available for this comparison.

merely bespeaks the failure in reproducibility of analyses of protein composition between laboratories and between samples, which is rather commonly encountered in the field of amino acid analysis.

The uncertainties in methods will undoubtedly become less in time, and this coupled with the increasing knowledge of average species requirements may be expected to provide more exact means of estimating the ability of a protein or mixture of proteins to fulfill specific functions. Even with the attainment of this goal, however, there will be continued dependence on animal assay methods if only because of their relative simplicity and directness.

2. Limiting Effects of Amino Acid Imbalance

The question of amino acid imbalance is large and complex and cannot be reviewed adequately here. That amino acid imbalance limits nutrition and may even lead to severe disturbances in metabolism and toxicity is well known. Methods of chemical scoring deal thus far only with adequacy of each essential amino acid and do not attempt to predict the effects of overabundance of single amino acids, or the effects of variations in proportions. There is sufficient variation in the dietaries of animals and groups of people and in the composition of individual proteins, which have been used extensively in nutrition studies, to warrant the idea that the body adjusts well to fairly large variations in the proportions of amino acids which are presented to it. On the other hand, even proteins of high nutritive value are known to foster varying responses in different tissues, as exemplified by the findings of Dr. Bacon Chow and described elsewhere in this symposium.

The extreme imbalance of certain proteins with regard to adequacy of the indispensable amino acids is heightened in some cases by the preponderance of certain amino acids which are actually inhibitory at high levels in the diet. This is notably true of gelatin and is related to the high glycine content of this protein, as shown by Hier, Graham and Klein (59).

Christensen and his coworkers (60–62) have presented new experimental evidence showing that the cells actually reject unbalanced amino acid mixtures. The studies show that the amino acid pattern of the cells can be upset by unbalanced amino acid levels in the extracellular fluid, leading to a loss of amino acids from the interior of the cells. This provides a rational explanation as to why certain acute amino acid imbalances such as that reported for high levels of glycine or methionine work to the nutritional disadvantage of the diet. These phenomena are without the present scope of chemical scoring.

V. Specificity of Amino Acid Requirements

1. Essential Amino Acids

Rose defines an "essential amino acid" as one which cannot be synthesized by the animal organism out of materials ordinarily available at a speed commensurate with the demands for normal growth. This concept was based on rat growth studies extending over many years. A final classification based on this work was presented recently by Rose, Oesterling and Womack (63). The classification for the requirements for maintenance of nitrogen balance in normal adult man as described by Rose (1) differs in that histidine and arginine do not appear to be needed by adult man. The adult dog differs from adult man only in the requirement for histidine, *cf.*, Rose and Rice (64). According to Frazier *et al.* (35), the adult rat requires the same 9 amino acids as the adult dog. The requirements of the chick for maximum rate of growth are more fastidious than that of either man or the rat according to Almquist and Grau (65). The 8 key amino acids required by all species under all conditions are: leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. In addition to these, the chick requires for maximum growth histidine, arginine, glycine and glutamic acid.

2. The Role of Nonessential Amino Acids and Other Sources of Nitrogen

The fact that glutamic acid and proline, like arginine, will stimulate growth rate in weanling rats fed a mixture of the 10 amino acids was indicated by the work of Womack and Rose (66). Later Rose *et al.* (63) showed that removal of glutamic acid from a mixture of 19 amino acids resulted in only slight inhibition of growth rate, which was statistically of doubtful significance. Still more recently Rose, Smith, Womack and Shane (67) reported the ability of diammonium citrate, glutamic acid, glycine or urea to provide the extra nitrogen, in addition to the 10 essential amino acid mixture, required for synthesis of the nonessential amino acids.

The recent work of Lardy and Feldott (68) is very clearcut, even though limited to only a few rats, in showing the complete dispensability of all but 10 amino acids for the growing rat. In these studies, an isonitrogenous amount of diammonium citrate was used to effectively replace the eight nonessential amino acids of the complete amino acid mixture (Mixture XXIII) of Rose *et al.* (63).

Work which has been progressing in this laboratory (69) by the method of liquid supplement feeding to adult protein-depleted rats confirms the above reports as to the ability of diammonium citrate to replace all of the nonessential amino acids. Unlike the young rat, the adult protein-depleted rat requires only 9 amino acids, responding maximally to isonitrogenous supplements of arginine, or glutamic acid, or diammonium citrate. In agreement with the findings of Rose *et al.* (67) for the growing rat, our findings in the adult protein-depleted rat place glycine in a position of intermediate effectiveness, and urea least efficient of all. In addition, Rose *et al.* (67) reported that ammonium acetate was fully as effective as diammonium citrate, thus ruling out the involvement of the citrate ion and the possible stimulation of this ion and glutamic acid to the Krebs-Henseleit tricarboxylic acid cycle.

The repletion-type studies in this laboratory wherein solutions of amino acids based on the composition of casein or on minimal mixtures were fed, as reported by the University of Chicago group, will be reported in detail elsewhere. For the purposes of the present discussion, it should be pointed out that the mixture of 9 essential amino acids in the proportions used by Steffee *et al.* (4, 75) was fed at a level to supply exactly twice the minimum amounts of each amino acid required to support a maximum rate of repletion. This is still a very low level of nitrogen, amounting to only 138 mg. N per rat day, of which 36 mg. was D-amino acid N of the unavailable isomers of isoleucine, threonine and valine. Thus, the amount of nitrogen physiologically available for repletion in these studies was only about 100 mg. per rat day, a highly critical allowance well below the level required for maximum rate of repletion. Despite this critical feeding level of total nitrogen, there was a fair response to the minima mixture alone. However, when about one-third of the total N of the minima mixture was replaced by diammonium citrate, arginine or glutamic acid, the rate of weight gain was increased more than 50 per cent. One-third isonitrogenous replacement with urea gave about a 25 per cent weight increase over the minima mixture alone. Thus it would appear that the ability of rats to convert the essential to nonessential amino acids is rather poor, and that the other sources of nitrogen mentioned are much more readily used by the body for this purpose. Results of replacement of part of the essential amino acid nitrogen by glutamic acid, arginine, urea, or diammonium citrate, are shown in Table V.

Foster, Schoenheimer and Rittenberg (70) had clearly demonstrated that dietary ammonium N^{15} is rapidly incorporated into rat tissue proteins. The above findings establish the utility of ammonia nitrogen in an entirely different way. In his general review of the role of the isotopes

TABLE V

Response of Adult Protein-Depleted Rats to the 9 Essential Amino Acid Minima Mixture Fed at Twice the Minimum Levels. Effect of Replacing Part of the Essential Amino Acid N by Various Nonspecific Nitrogen Sources

Mixture Fed	Av. 12-day weight gain, g.	Standard error, g.
Minima mixture alone (1st assay)	19	±2.2
Minima mixture (Repeat assay in same rats)	22	
Minima with 19.3 per cent arginine N (1st assay)	34	±2.0
Minima with 19.3 per cent arginine N (Repeat assay)	32	
Minima mixture alone	21	±2.6
Minima with 32.4 per cent Urea N	26	±2.4
Minima with 32.4 per cent arginine N	35	±2.3
Minima with 32.4 per cent glutamic acid N	36	±1.9
Minima mixture alone	23.2	±1.9
Minima with 10 per cent ammonium acetate N	34	±3.5
Minima with 20 per cent ammonium acetate N	36	±4.3
Minima with 30 per cent ammonium acetate N	43	±8.4

Four sets of experiments are shown. Groups of 5 to 6 rats were used in each experiment. All solutions were made to contain .55 to .65 per cent nitrogen. Standard nitrogen allotments of .138 g. N per rat day in 25 cc. were fed throughout. This level of nitrogen provided twice the amounts of Cannon's minima for each of the essential amino acids in the case of the minima mixture alone. Replacement of part of the nitrogen of the minima mixture by other nitrogen sources was made, as shown.

to reveal the dynamic state of body constituents, Schoenheimer (71) reported the virtually complete excretion of dietary urea N^{15} in unchanged form. It is revealing then, from the above work, that under conditions of stress, the body can actually use urea to some extent as a source of nitrogen, and can use urea nitrogen even more efficiently than essential amino acid nitrogen for general synthetic purposes. In the adult protein-depleted rat as in the growing rat, there is a wide range of nitrogen compounds other than the nonessential amino acids which would need to be synthesized from dietary nitrogen. Obviously, these compounds can all be synthesized physiologically from ammonia and carbon residues derived from normal metabolism cycles.

It is now well established that two chief reactions occur in the tissues with regard to changes in the nitrogen moiety: (1) transamination and (2) oxidative deamination and subsequent urea formation. It is also fairly well established that alanine and glutamic and aspartic acids are the only amino acids which enter readily into transamination reactions with the α-keto acids known to occur in the body. Glutamic acid is

an efficient source of nitrogen to replace all other nonessential amino acids, and by the same token, it is expected that alanine and aspartic acid would serve well in the same capacity. On the other hand, none of the essential amino acids enter readily into this reaction and, therefore, must be degraded principally by oxidative deamination. There is evidence that the latter reaction occurs in the liver and leads directly to urea formation. In the body economy one might expect that reaction mechanisms would be in the direction of preserving the essential amino acids, rather than degrading them rapidly, and this, indeed, appears to be the case.

The beauty of the isotope technique to trace the metabolic fate of amino acid N is exemplified by a recent paper by Wu and Rittenberg (72) in which the metabolic fate of L-aspartic acid is described. The findings suggest that aspartic acid is so rapidly deaminated that its amino group behaves metabolically like ammonia. In regard to the isotope studies, it may be worthy of note that the first broad interpretation was that all L-amino acids are readily deaminated. This idea is apparent in the extensive review of the role of the dicarboxylic acids in nitrogen metabolism by Braunstein (73). On the contrary, the nutritional studies in this laboratory with protein-depleted rats clearly support the idea that the deamination of essential amino acids is quite limited. One deals both with equilibria and rates of reaction, and, coupled with these factors in utilization from the nutritional viewpoint, is the factor of renal excretion. For example, normal animals on normal diets excrete urea almost quantitatively, *i.e.*, the urea clearance test, whereas animals receiving only the essential amino acids can use urea to fairly good advantage. Furthermore, in balance studies, these animals did not excrete much more urea than did rats receiving only the 9 essentials. Rats receiving one-third of their total nitrogen intake in the form of diammonium citrate did not excrete appreciably more ammonia and urea nitrogen than rats on normal diets. This confirms the similar findings of Lardy and Feldott (68) with growing rats with 10 and 19 amino acid mixtures in which diammonium citrate was successfully used to replace the nonessential amino acids. Results from three laboratories thus confirm the finding that diammonium citrate can completely replace the nonessential amino acids in amino acid mixtures.

3. The Role of Arginine

The situation with regard to arginine requires special consideration. As first reported by Frazier *et al.* (35), rats receiving only 9 essential amino acids did as well as animals receiving a 16 amino acid mixture

patterned after casein, or the 10 amino acid mixture containing arginine. This is contrary to our experience, as we have consistently found that almost any source of other than essential amino acid nitrogen will serve to improve the response over that shown by the mixture of 9 essentials alone. Following the work of Frazier *et al.*, it was noted in the joint work of Wissler *et al.* (37) that the addition of arginine to a 9 amino acid mixture did stimulate appetite in normal adult animals.

A possible explanation of the failure of Frazier *et al.* (35) to show an effect of the nonessential amino acids over and above that of the 9 essentials is found in the comparative amino acid mixtures used by these authors. The mixture of 16 amino acids patterned after casein contains a rather low level of methionine and only a trace of cystine. One would expect the sulfur amino acids to be limiting, as in casein. In the comparative feeding experiments, however, the rats received a much higher level of methionine from the 9 than from the 16 amino acid mixture. Thus, the nutritional advantage related to higher methionine level in the mixture of only the essentials may have balanced the nutritional advantage which we would ascribe to the presence of the nonessential amino acids in the 16 amino acid mixture.

The role of arginine in the nutrition of the growing rat has been a subject of continued study at the University of Illinois for many years. The results of the more recent studies of Borman *et al.* (74) confirmed the earlier evidence as to the requirement of arginine for maximum growth in the rat. The diet used was much improved with regard to purity and adequacy of vitamin supplements over diets formerly available, so that average growth rates were greatly increased and the effect of arginine was clearcut. More recently Womack and Rose (66) demonstrated the fact that either glutamic acid or proline could partly substitute for an isonitrogenous addition of arginine. The findings were interpreted "as evidence that the three amino acids are mutually interconvertible in the organism of the rat, but at different rates as exemplified by their different influence upon growth." In view of our recent findings in the adult rat, one would say that glutamic acid and arginine, at least, are readily used as sources of ammonia N for conversion to all other nonessential amino acids. Emphasis was placed in the report of Womack and Rose (66) on the essentiality of arginine, and the relation of glutamic acid and proline to arginine. The requirement of the growing rat for arginine appears to be well established from the work of Rose and his collaborators. It would be of interest, in the light of recent work, to determine whether or not there is a sparing effect of fairly high levels of glutamic acid and diammonium citrate for arginine in the growing rat.

4. The Role of Glutamic Acid

The recent experiments of Rose, Oesterling and Womack (63) showed that in 28 days rats receiving only 10 amino acids gained about 70 to 75 per cent as much as litter mates which received 19 amino acids. Although addition of glutamic acid to the 10 amino acid mixture stimulated growth, removal of the glutamic acid from the 19 amino acid mixture did not have a statistically significant effect. The authors stated further that under similar conditions the influence, if any, of glutamic acid upon growth is much less than that manifested by arginine. In view of these facts, glutamic acid was classified as a dispensable dietary component for the rat, and this conclusion is well supported by the work cited from other laboratories.

Establishment of the complete dispensability of glutamic and aspartic acids for the nutrition of the rat is of interest with regard to the preparation of intravenous amino acid mixtures, wherein the presence of a high proportion of these amino acids is undesirable.

5. Ratio of Essential Amino Acid N to Nonspecific N

On the basis of experiments in this laboratory, it would seem that the ratio of essential to other than essential amino acid N is of primary importance when studying the efficiency of any given source of other than essential amino acid N. Although the optimum level of other than essential amino acid N has not been determined accurately, it is clear that one-third substitution of the total nitrogen in the form of glutamic acid, arginine, or diammonium citrate supports an excellent response. The effect of these sources of nitrogen is very pronounced at the above level. Very significant effects were noted also for arginine, glycine, and a mixture of glycine and alanine at one-fifth of the total N. Thus, it is clear that small additions or substitutions of various sources of nitrogen to the essential amino acid mixture would all be expected to produce a supplementary sparing effect, up to an undetermined optimum ratio.

6. Experimental Approaches Used by Three Different Laboratories

The conditions used by the 3 different laboratories in showing the utilization of sources of other than essential amino acid nitrogen are all quite different and are, therefore, difficult to compare. Rose *et al.* (67) measured the stimulation of growth of young rats when other sources of nitrogen were added to the mixture (Mixture XXVI) of the 10 essential amino acids fed at the predetermined minimum levels, *i.e.*, 8.82

per cent of the diet. Under these conditions of additive effect, glutamic acid and diammonium citrate produced the greatest growth, glycine was next, and urea poorest. In another type of experiment, these authors fed only one-half the minimum levels of the 10 essentials to 3 young rats and measured the effects of additions on the maintenance of weight and nitrogen equilibrium. Only the L-amino acid forms were used in this experiment (Mixture XXVII), and the mixture comprised only 3.46 per cent of the diet. Supplements of urea or diammonium citrate were made to supply an amount of nitrogen equal to that of the amino acid mixture. Under these conditions, both supplements induced strong nitrogen retention accompanied by some weight increase.

Lardy and Feldott (68) compared weight gain and N-retention of young rats on: (a) the 18 amino acid mixture (Mixture XXIII) of Rose *et al.* (63) at 10.3 per cent of the diet; (b) the 10 essential amino acid mixture at 8 per cent of the diet, and (c) the 10 essential amino acid mixture at 8 per cent and diammonium citrate (2.15 per cent) to equal the nonessential amino acid N of the 18 amino acid mixture. Mixtures of the 10 L-amino acids and the same plus diammonium citrate were also studied. The level of essential physiologically active amino acids in the diets was 6.4 per cent. The data presented showed that the diammonium citrate replaced all of the nonessential amino acids in stimulating growth rate above that shown with the 10 amino acids alone. The balance data further showed that urinary ammonia N loss following diammonium citrate feeding was not far greater than that following amino acids alone. With regard to the ratios of the different forms of nitrogen, it was of interest to calculate that 19 per cent of total N of the mixture plus diammonium citrate consisted of NH_3–N, and 30.7 per cent of total N was present as NH_3–N plus arginine N.

The experiments in this laboratory with adult protein-depleted rats, as compared with the experiments with growing rats, involved controlled rather than *ad libitum* amino acid feeding. The essential amino acids were offered at twice the levels required for rapid recovery. Substitution of a part of the nitrogen of the essentials by various other sources of nitrogen elicited a much greater response than that given by the essential amino acids alone. Thus, it became clear that the essential amino acids themselves are not readily available as sources of nitrogen for conversion to other nitrogen components of the body. The conversion of methionine to cystine and of phenylalanine to tyrosine appears as an exception to this generalization. This failure in ready conversion of the essential amino acids to other general forms of nitrogen may represent a real difference in metabolism of the two classifications. There is, of course, good reason for the body to conserve essential amino acid nitrogen, if

one wishes to invoke purely teleological reasoning. It is, nevertheless, surprising that the body can utilize urea even more efficiently as a nonspecific N source than it can a complete mixture of the essential amino acids.

7. Significance of Recent Findings

The concept that rats can readily synthesize all of the nonessential amino acids from a variety of nonspecific nitrogen sources has crystallized only recently. The previously held concept has been that the task of synthesizing the 10-odd nonessential amino acids simultaneously would present too great a physiologic burden for maximum rate of growth to proceed. Further, the fact was not known that synthesis of nonessential amino acids from an excess of essential amino acids proceeds slowly, and does indeed present a physiologic burden. Coupled with this was the rather firmly held idea, based on certain experimental evidence, that monograstic animals are entirely incapable of utilization of simple sources of nitrogen, such as ammonia and urea. The finding then that such simple forms of nitrogen as ammonium salts, urea and various of the nonessential amino acids may actually serve better than the essential amino acids themselves for general synthetic processes will come as a surprise to many.

On the other hand, it has long been known that proteins contain a significant amount of amide nitrogen, as glutamine and asparagine. Expressed as ammonia N, this form of nitrogen represents about 8 to 12 per cent of most food proteins. Although these forms of potential ammonia N are probably absorbed as such from the gastrointestinal tract, it is well known that they are involved in labile reversible systems within the body.

Almquist (76) has presented an excellent review of the amino acid requirements of avian species. He has also been concerned with the best definition of an "essential amino acid" and defines it as "one which must normally be obtained from the gastrointestinal tract in order that synthesis of body proteins may take place." On the basis of experiments in his own laboratory (77), Almquist has classified glutamic acid, together with arginine and glycine and the 9 amino acids required by the rat, all as requirements for normal growth of the chick. In light of the recent findings with the rat, it would be of considerable interest to determine whether or not simple nitrogen sources of ammonia can serve the chick equally as well as glycine, glutamic acid and arginine when all of the essential amino acids are supplied at adequate levels in the diet.

VI. Racemic Amino Acid Mixtures

A major problem in both the experimental and developmental use of mixtures of synthetic amino acids comes in the fact that the unnatural D-forms of certain of the essential amino acids are not utilized. Unfortunately, this is true for certain of the most expensive and rare amino acids, namely, threonine, isoleucine and valine.

Tryptophan presents a special problem in that the DL-forms are both used by the rat and the dog although the D-form may be less well utilized than the L-form. Only L-tryptophan is used by the human according to Rose (1) and Heber and Berg (81). Thus, assays of protein preparations with rats may not give a clear prediction of their value for human use with regard to the content of this amino acid. This circumstance deserves special consideration because of the relative instability of tryptophan to chemical treatments, such as acid hydrolysis.

Rose (1, 82) has discussed the activity of the D and L forms of each of the essential amino acids for the rat and the human. A review of the literature pertinent to the activity of the optical isomers of each of the amino acids for various species will not be attempted here. It would appear, however, from the results to date that the only significant discriminatory difference in utilization by rats and man is found in the case of tryptophan, as discussed above. Neither species, according to Rose, can utilize the D-isomers of valine, leucine, isoleucine, threonine and lysine. Both species can use racemic methionine fully, and can make partial inversion of D-phenylalanine.

1. The Question of Inhibition by D-Amino Acids

One might expect that the unusable isomers of the amino acids would show some interference in the normal metabolism of their usable enantiomorphs. That this is indeed true, at least in the case of D-valine and D-leucine, has been shown by Fling and Fox (83) in experiments with bacteria. D-alanine was not inhibitory, and this was explained theoretically on the basis that the methyl group of alanine is sufficiently small that it would not intervene, or cause steric hindrance, as is thought to be the case for the larger side chains of leucine and valine. The antipodal specificity of proteolytic enzymes is well known and it would not be surprising if the inhibition caused by certain of the D-amino acids comes at that point. The further illuminating finding has been reported by Prescott, Schweigert, Lyman and Kuiken (84) that DL-tryptophan is not as effective in meeting the growth requirements of certain microorganisms as a one-half level of L-tryptophan. The presence of D-tryp-

tophan in this case made for an increased requirement for L-tryptophan, a situation common to many reversible competitive inhibitions.

Evidence for the inhibitory effect of D-amino acids in animals is less clear than that for bacteria; however, the preponderance of evidence indicates that competitive inhibition does take place. Wretlind (85) reported that a mixture of 10 DL-amino acids patterned after Rose's minimum requirements for the rat gave a greater growth response at a level of 20 per cent than at 30 or 40 per cent in a purified diet. The results were interpreted as meaning that completely racemic substances contain toxic substances. Because Wretlind did not supply any source of other than essential amino acid nitrogen in the diet, there remains the possibility that the inhibition was caused by an overloading of the capacity of the body to convert essential amino acid nitrogen to nonessential amino acids and other nitrogenous compounds necessary for growth. This possibility, however, appears remote, and may be checked by experiments in which a readily used form of nonspecific nitrogen is included in the diets.

The presence of racemic valine, isoleucine, threonine and phenylalanine in 3 of the mixtures studied by Lardy and Feldott (68) did not appear from their data to inhibit intake or weight response as compared with mixtures of only L-amino acids. On the other hand, Brand and Bosshardt (86) reported that a mixture of L-amino acids duplicating the composition of β-lactoglobulin supported the same growth in mice as a corresponding amount of the whole protein, whereas use of certain DL-amino acids in the mixture impaired the growth response. It has been repeatedly observed in this laboratory in the aforementioned rat-repletion studies that amino acid mixtures containing the racemic forms of valine, isoleucine, and threonine are accepted with much less avidity than are complete hydrolysates of fibrin supplemented only with DL-tryptophan. This is true even when adequate sources of other than essential amino acid nitrogen are supplied. A very striking difference is the slow rate at which the rats consume the amino acid solutions compared with the eager and rapid drinking of hydrolysate solutions. The crucial experiment to finally answer this point must await the preparation of a mixture of the 10 essential L-amino acids in fair quantity. The suggestion is clear, however, that certain of the D-amino acids exert inhibitory effects on metabolism in various forms of life.

Albanese *et al.* (87) have recently presented the thesis, based on certain indirect evidence in work with human infants, that DL-tryptophan may have some untoward effects not shown by L-tryptophan when used as supplements to tryptophan-low diets. There is need for a critical and fundamental study in animals of the effects of DL-tryptophan as

compared with L-tryptophan. The addition of DL-tryptophan to parenteral amino acid solutions is now common practice and no gross aberrant effects have been noted. The question is a complex one, however, and may require more basic study than it has received.

In comparing rat growth on mixtures of amino acids with casein and casein hydrolysates, Ramasarma, Henderson and Elvehjem (88) noted slower growth on the former. One possible explanation which they advanced for the slower growth on the amino acid mixtures was the presence of D-amino acids. As stated, "at the levels used, they may not produce any symptoms of toxicity but may cause a slight depression of growth."

During final reading of this review, a paper appeared by Van Pilsum and Berg (89), which bears directly on the effects of the D and L forms of amino acids on growth. The authors reported that the L forms of the ten essential amino acids as components of a DL mixture constituting 22.4 per cent of the diet grew less well than control rats fed only the L isomers at a dietary level of 11.2 per cent. However, when only half as much DL-phenylalanine, tryptophan, methionine and arginine, and an intermediate level of DL-histidine were included, the resulting 18.6 per cent of DL-amino acids promoted as good growth as that attained on the L mixture.

The interesting finding was then made that removal of half of the DL-methionine overcame the growth-retarding effect of the 22.4 per cent DL mixture. Comparative tests showed that the growth retardation produced by the natural L isomer of methionine was greater than that produced by either the DL or the D modification. The toxicity of excess methionine is well-known. Both forms of methionine can be used, and it is possible that an excess of L-methionine exerts more toxicity than the D form because it is more readily utilized. This situation, of course, may not apply for those amino acids for which the D-amino acid is not used.

Van Pilsum and Berg concluded that "contrary to the often repeated conjecture that the D forms of the essential amino acids may be toxic in the rat, proportionately large amounts can actually be fed as components of DL-amino acid mixtures without producing any apparent growth retarding or other deleterious effect." Although it is clear from these experiments that the D-amino acids are for the most part inert, certain questions require investigation before the argument can be entirely closed regarding their reputed inhibitory effects. It will be noted that in the above cited work, growth rates were slower on both the L and DL-amino acid mixtures than on properly fortified casein hydrolysates. This is similar to the finding in this laboratory (69) that protein hydrol-

ysates were accepted with much greater avidity by protein-depleted rats than any of the amino acid mixtures studied, all of which contained at least four racemic amino acids. In lieu of any better explanation, the idea was advanced at that time that the difference in acceptance might be related to the presence of D-amino acids in the amino acid mixtures.

An important consideration in interpretation of the work of Van Pilsum and Berg is the absence of non-specific nitrogen from their amino acid mixtures. Although these authors added ammonium citrate and glycine to their essential amino acid mixtures in a few studies, the additions did not appear to have any effect on growth, and the question was not examined further. The absence of a sufficient concentration of other than essential amino acid nitrogen would, however, be expected to inhibit response to a measurable degree, judging from the work of Lardy and Feldott (68), as well as the work in this laboratory. The possibility appears, therefore, that there may have been a second variable at work, other than the optical form of the amino acid groups. Judging from all of the evidence, it would seem desirable to have present in such a study an adequate source of other than essential amino acid nitrogen so that synthesis of non-essential N compounds could proceed more readily than is the case when only essential amino acids are present. The excellent study of Van Pilsum and Berg points up the need for this additional experimental approach to the effect of D-amino acids.

2. Present Implications

There are many important implications in the knowledge of the effects of the racemic amino acids as regards medical and commercial interest in amino acids. The work herein reviewed points up the likelihood that mixtures of only 8 or 9 amino acids plus a source of ammonia nitrogen would function efficiently as a medical tool for prevention and correction of hypoproteinemia. Rose, Smith, Womack and Shane (67) have made the further significant disclosure that the α-keto analogues of valine and isoleucine promote excellent growth in rats as the sole sources of these amino acids. The suggestion is made that they undergo asymmetric amination in vivo to supply the corresponding L-amino acids. If the α-keto analogs are less costly to synthesize, strategic use might be made of them, together with a source of ammonia, in idealized mixtures.

VII. Summary

An attempt has been made to briefly review methods of assay of protein value, with particular regard to those methods which have been

used in recent years to assay liquid protein hydrolysates intended for therapeutic use. Space has not permitted consideration of all of the literature and methods which deserve attention, and greatest emphasis has been placed on the rat-repletion method.

The problem of devising a rapid and accurate control method for assessing the nutritive value of intravenous protein hydrolysates is shown to be a complicated one. Although the rat-repletion method serves to establish amino acid adequacy, it does not fully predict the availability of a preparation when given intravenously. Methods based on injection procedures in adult animals depend on maintenance of nitrogen balance, rather than growth or tissue regeneration. The fundamental accuracy of short-term nitrogen balance studies in adult animals in resolving differences in nutritive value has been a moot question for some time. One solution to the problem would be the establishment of intravenous utilization of a specific hydrolysate by long-term nitrogen balance and regeneration studies, followed by routine control of essential amino acid adequacy by a growth or repletion method.

An attempt has also been made to compare the amino acid requirements of the rat with those of the human, and to estimate the value of proteins in terms of amino acid composition. The limitations of chemical scoring have been briefly discussed.

Considerable attention is given to the nutritive role of the nonessential amino acids, which has recently been clarified in 3 laboratories. The evidence indicates that none of the nonessential amino acids plays a highly specialized nutritive role, and that various sources of nitrogen can serve to meet the needs of other than essential amino acid nitrogen. There is clear evidence that the essential amino acids are not readily metabolized to provide sources of nitrogen for synthesis of the nonessential amino acids. Diammonium citrate, ammonium acetate, glutamic acid and arginine serve equally well as sources of non-specific nitrogen to substitute for the nonessential amino acids. Urea is less efficient, but under the purely artificial conditions imposed in these experiments is used to a surprising degree in sparing essential amino acid nitrogen.

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The Laboratory Evaluation of Amino Acid Mixtures and Protein Hydrolysates

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CONTENTS

I. Introduction 75
II. Experimental 77
1. Weight Repletion Experiments 77
2. Liver Protein Repletion Experiments 78
3. Acceptability of Amino Acid Diets in Short-Term Studies 82
4. Nitrogen Balance Studies 85
a. Adequacy of Proteins and Protein Hydrolysates 85
b. Nitrogen Retention After Feeding Deficient Amino Acid Mixtures and the Effect of Supplying the Deficient Amino Acid Previously . 88
c. Nitrogen Retention After Intraperitoneal Administration of Deficient Amino Acid Mixtures 89
d. Imbalance Effect on Nitrogen Retention 90
III. Discussion 91
References 92

I. Introduction

Most of the available information regarding the utilization of proteins or protein derivatives has been obtained in animal growth, repletion, or nitrogen balance studies. Each of these procedures is capable of yielding valid data regarding utilization relative to a specific function, such as increase in body weight, replacement of liver protein, replacement of serum protein, or overall retention of nitrogen. However, no single procedure gives a complete picture of the utilization of a given protein or hydrolysate, and in comparative studies the results are not infrequently complicated by variations in caloric or nitrogen intake, particularly in those experiments in which part or all of the diet is supplied *ad libitum*. The *ad libitum* feeding of diets containing substances to be tested for biological value introduces the teleological line of reasoning that, if the substance is nutritionally adequate, it will be consumed and utilized, and if one diet is voluntarily ingested in larger amounts and thereby is more effective in promoting body weight or tissue, it contains the superior protein. The validity of this concept is open to question.

Mitchell and Beadles (1) have stressed the necessity for comparable intake of protein diets in the comparison of the nutritional value of proteins. In nitrogen balance studies also, caloric intake as well as nitrogen intake must be carefully controlled for accurate evaluation. The mere demonstration of positive or negative balance is insufficient. Previous dietary history greatly influences nitrogen balance so proper control studies, employing protein-free diet or a protein of known quality, are imperative.

In the laboratory study of preparations designed for intravenous administration to man, even more complications are encountered. In addition to possible differences in species requirements, and differences in physiological conditions (related to growth, age, deficiency, or disease) it must be recognized that the requirements for amino acids infused intravenously cannot be identical to the requirements for amino acids fed orally. Differences in rates of utilization, destruction, and excretion after intravenous feeding require that the "ideal" parenteral amino acid solution be unlike the "ideal" oral preparation. Even greater differences should be anticipated between the composition of the "ideal" protein and the "ideal" parenteral amino acid solution due to the additional factors of rates of digestion and absorption.

However, since our knowledge regarding the specific amino acid needs of individual patients [1] is practically non-existent and thorough evaluation under clinical conditions is difficult, if not impossible, much of the development and control of parenteral solutions is of necessity carried out with laboratory animals. The experiments reported here have been planned with the primary objective of finding the least undesirable laboratory test for amino acid mixtures and protein hydrolysates, designed for the intravenous feeding of man.

As early as 1944, the studies of Kosterlitz (4, 5) led us to investigate the replacement of liver protein as a measure of the biological value of amino acid mixtures. When a study of the effects of deficient amino acid mixtures was attempted, however, refusal of the protein-depleted rat to consume adequate amounts of the incomplete diets made interpretations difficult. In 1947, an attempt was made to apply a repletion test (6) to the problem, but again inadequate food intake was a complication. Forced feeding of sufficient amounts of amino acids for growth in rats was unsuccessful, due primarily to the development of diarrhea. Finally, in 1948, the nitrogen balance approach (7, 8) was investigated. The

[1] The authors have proposed that a modified saturation test approach might be applied to this problem. Comparison of the amino acid excretion pattern with the pattern of infused amino acids should reveal changes or differences in amino acid requirements (2, 3).

measurement of nitrogen balance after intraperitoneal administration of amino acid solutions to rats seemed to show promise, so a series of experiments was conducted to apply such testing to a variety of amino acid mixtures. This presenation will deal almost exclusively with hitherto unpublished data from the Merck Institute.

II. Experimental

1. Weight Repletion Experiments

Male rats weighing about 235 g. each were fed a protein-free diet [2] for 3 weeks. Five rats were each then offered 15 g. of diet per day after the test protein, amino acid mixture,[3] or protein hydrolysate had been incorporated in it at a concentration of 1.3 per cent nitrogen. The rats were weighed after 4 days and again after 14 days subsequent to overnight fasting, and liver total nitrogen determinations were made so that the body weight responses could be correlated with the replacement of liver protein. The data are shown in Table I.

TABLE I

Repletion Experiment with N-Source Incorporated in Diet

N-Source	Wts., g. Initial	Wts., g. 4 days	Wts., g. 14 days	Av. wt. gain	Av. intake/day, g. 1st 4 days	Av. intake/day, g. 5-14 days	Av. liver wt., g.	Liver N mg./100 g. wt.
——	192	188	175	—17	11.6	12.3	5.3	85±3.6[b]
A	192	204	227	35	13	15.2	6.4	104±1.8
BB	193	198	208	15	10.5	13.4	5.2	94±1.5
D	196	206	225	29	12.2	14.6	6.2	101±3.1
E_1	193	202	218	25	12.6	14.6	6.0	102±3.6
Casein	192	207	236	44	13.5	15.4	6.7	104±2.4
Gelatin	193	189	175	—18	10.1[a]	10.4[a]	4.3	82±2.0
Lactalbumin	192	213	241	49	14.1	15.1	7.3	106±4.8

[a] Probably higher than true consumption due to excessive loss through spillage.
[b] Standard error.
A, BB, and D protein hydrolysates of commercial origin.
E_1 amino acid mixture of the VujN type (10).

[2] Per cent composition of protein-free diet: cornstarch, 70; hydrogenated vegetable oil, 24; salt mixture USP I, 4; cod liver oil, 2; thiamine HCl, 0.001; pyridoxine HCl, 0.001; riboflavin, 0.002; calcium pantothenate, 0.010; nicotinamide, 0.010; inositol, 0.050; choline chloride, 0.100.

[3] Per cent composition of VujN mixture E_1: L-arginine, 2.34; L-histidine, 2.55; L-isoleucine, 7.67; L-leucine, 15.90; L-lysine, 10.60; L-methionine, 4.37; L-phenylalanine, 4.15; L-threonine, 1.38; DL-tryptophan, 1.70; L-valine, 6.92; L-glutamic acid, 0.43; L-tyrosine, 0.21; glycine, 24.50; lactic acid, 10.60; unidentified amino acids, 6.68.

In a second experiment, groups of 5 rats were offered solutions of hydrolysates and one amino acid mixture in place of water as suggested by Frost and Sandy (9). In other respects, this experiment was similar to the previous one. Each rat was offered daily portions of 33 cc. of solutions containing 200 mg. of total nitrogen. The data are summarized in Table II.

TABLE II

Repletion Experiment with N-Source in Drinking Water

N-Source	Wts., g.		Av. wt. gain	Av. intake/day	
	Initial	14 days		g. diet	cc.
——	219	197	—22	8.7	13
A	211	246	35	10.4	32
B	218	258	40	10.5	33
C	220	242	22	10.0	33
D	218	246	28	10.4	27
E_2	218	223	5	8.3	19

Preparations A and D were used in both experiments and the results were almost identical. B and BB were different batches of one type of protein hydrolysate. E_1 and E_2 were almost identical preparations of the VujN type (10). The response to E_1 in the diet was more satisfactory than the response to E_2 in solution. When the solution of E_2 was supplied in place of water, the rats consumed less than two-thirds as much water and nitrogen and only 80 per cent as much protein-free diet as the rats fed A, B, or C. The factor of acceptability appeared to be more critical in the second experiment when the amino acid mixture was offered in solution, rather than incorporated in the diet. There can be no doubt concerning the nutritional adequacy of the amino acid mixture. In nitrogen balance studies in rats this mixture was at least equal to casein, and a similar mixture, E_1, promoted growth when fed in the diet and even the small amount which was taken in liquid form prevented a loss of weight; in other words, there was evidence of utilization of the nitrogen consumed. Whether the solution of amino acids was distasteful to the rats, or produced a feeling of discomfort upon ingestion, cannot be determined from the present evidence. This point will be reconsidered in a later section.

2. Liver Protein Repletion Experiments

After adult rats consumed a protein-free diet for 3 days and then a diet containing casein for 2 days, the amount of liver protein (as indicated by total N determination) varied in proportion to the concen-

tration of protein in the diet. Twelve g. of diet per 200 g. rat were fed on the first day of repletion, 10 g. on the second. The results of this study are shown in Table III. In Table IV are summarized similar

TABLE III

Repletion of Liver Protein After Feeding Various Concentrations of Casein in the Diet for 2 Days Following a 3-Day Protein Depletion Period

Per cent casein	No. of rats	Rat wts., g.		Liver		
		initial	final	Wt., g.	Total N, mg.	Mg. N/100 g. rat
0	15	209	199	7.06	198	90±1.5
5	8	226	210	7.03	210	93±2.1
7.5	6	218	206	6.66	237	109±9.0
12	8	206	200	7.27	231	112±2.3
15	48	205	200	7.28	236	115±0.9
18	8	204	200	7.26	240	123±1.9
21	8	226	221	8.60	289	128±2.1
24	8	206	206	7.93	270	131±2.3
28	8	221	216	8.60	299	135±1.6
30	8	212	208	8.55	287	137±3.7
33	8	202	207	7.14	282	139±2.2
38	8	230	228	9.83	334	145±2.2
40	6	217	218	9.46	297	138±3.5
43	8	222	219	9.61	326	147±3.5

TABLE IV

Repletion of Liver Protein After Feeding Various Concentrations of Casein in the Diet for 2 Days Following a 4-Week Protein Depletion Period

Per cent casein	No. of rats	Rat wts., g.		Liver			Serum protein, g. per cent
		initial [a]	final	Wt. g.	Total N, mg.	Mg. N/100 g. rat	
0	15	169	164	6.26	142	84±1.1	4.44
7.5	6	162	164	7.21	166	102±2.8	4.89
15	12	173	180	6.68	193	111±1.6	5.14
20	6	183	190	7.14	226	122±3.2	5.23
30	6	162	175	7.89	248	152±3.9	5.42
40	5	160	171	7.69	271	169±6.6	5.73

[a] Initial weight taken as weight 3 days before feeding test diet, to be comparable to 5-day test.

data obtained after a 4-week depletion period. In this experiment, serum protein concentrations were also determined.

These data are in agreement with those of Kosterlitz and illustrate

the dependence of liver total nitrogen upon the amount of protein ingested. It is obvious that the liver nitrogen response must also depend upon the amino acid pattern of the protein fed, and that a suboptimal content of any one or more essential amino acids may inhibit that response.

There was no difference in repletion of liver protein between rats depleted 3 days or 4 weeks when 7.5 per cent to 20 per cent casein diets were fed. However, the more severely depleted rats showed a greater capacity for liver protein synthesis when higher concentrations of dietary casein were supplied. The serum protein concentrations also increased with the protein content of the diet.

A number of protein samples and 3 enzymatic digests of proteins were also studied in rats depleted either 3 days or 4 weeks (Table V).

TABLE V

5-Day Test of Liver Protein Repletion

N-Source	No. of rats	Rat wts., g. initial	Rat wts., g. 5 days	Liver Wt., g.	Liver TN, mg.	Liver mg. N/100 g. rat	Serum protein g. per cent
Zein	6	184	173	5.94	169	92±2.1	
Gelatin	8	214	198	7.90	208	97±2.4	
Dried yeast	6	184	178	6.03	182	99±3.7	
Dried beef liver	5	182	174	6.68	194	106±3.0	
Soya meal	6	185	178	6.13	197	107±3.9	
Egg albumin	5	183	180	6.29	198	108±1.9	
Egg powder	6	184	179	6.17	204	112±2.8	
Lactalbumin	8	202	199	8.51	261	128±4.5	
Yeast digest	8	200	200	7.19	216	106±2.0	
Casein digest	12	208	208	7.73	237	112±2.1	
Lactalbumin digest	8	215	208	8.14	253	117±1.9	
30-Day Test of Liver and Serum Protein Repletion							
Zein	6	173	165	6.68	155	89±2.9	4.76
Gelatin	6	171	166	6.06	152	89±2.2	4.43
Dried yeast	6	176	176	6.82	179	102±3.5	4.81
Dried beef liver	6	171	176	6.98	186	108±4.7	5.41
Soya meal	6	171	171	5.81	155	91±4.5	5.38
Egg albumin	6	173	179	6.86	193	112±4.6	5.38
Egg powder	6	175	176	6.90	191	109±4.7	5.44
Lactalbumin	6	172	179	7.57	216	126±2.6	5.40
Yeast digest	7	171	173	6.94	171	109±1.1	5.31
Casein digest	8	165	170	6.58	180	110±2.5	5.17
Lactalbumin digest	6	168	174	7.34	200	119±3.5	5.52

The materials were incorporated in the diet at a concentration equivalent to 15 per cent casein (2.1 per cent N) and each rat was offered 12 g. of diet per 200 g. rat the first day and 10 g. the second, as in the previous experiments. Serum protein determinations were again made on pooled samples from the rats which had been depleted of protein for 4 weeks prior to the 2-day repletion period.

The greater response of liver nitrogen to lactalbumin feeding is in agreement with the accepted nutritional superiority of this protein (11). Defatted whole egg powder was approximately equal to casein, while egg albumin, dried beef liver and dried yeast were about half as effective. As predicted, zein and gelatin were inferior. The digest of lactalbumin was less effective than the intact protein. The casein digest was of slightly less nutritional value than casein.

Certain materials (such as liver, soya meal, egg albumin or powder, and yeast digest) which were inferior or equal to casein in the promotion of liver protein regeneration were superior in stimulating serum protein synthesis. These data illustrate the thesis previously mentioned, that any one test of biological utilization leaves much to be desired from the overall point of view. A protein that preferentially promotes liver protein synthesis may be more desirable under certain conditions (such as liver disease) whereas one that favors serum protein regeneration may be more appropriate under other conditions (such as hemorrhage).

The effect of diets deficient in single amino acids was also studied. In the 5-day liver repletion experiment, the Vuj[4] amino acid mixture of Madden and Clay (12) or the same mixture with single amino acids omitted was administered orally in 2 portions on each of the 2 days of repletion. The nitrogen intake was calculated to equal the total nitrogen intake when 15 per cent casein diet was fed, as described above. The results are shown in Table VI.

Replacement of liver nitrogen was noted in all animals given amino acids, whether the mixture contained all 10 "essential" amino acids or only 9. However, the methionine-free mixture was least efficient. The mixture lacking arginine promoted more liver protein formation than the complete mixture. Omission of arginine, histidine, or leucine appeared to have no inhibiting effect on liver protein formation in these experiments. The effects of deprivation of arginine and methionine were confirmed in rats after a 4-week depletion period.

It appears that the rat is able to synthesize, or mobilize from other

[4] Per cent composition of Vuj amino acid mixture: L-arginine HCl, 8.0; L-histidine HCl-H_2O, 4.0; DL-isoleucine, 10.8; L-leucine, 15.4; L-lysine HCl, 12.3; DL-methionine, 6.1; DL-phenylalanine, 6.9; DL-threonine, 10.8; DL-tryptophan, 1.8; DL-valine, 13.9; glycine, 10.0.

TABLE VI

Liver Protein Repletion of Rats Fed Amino Acid Mixtures
5-Day Test

Mixture	No. of rats	Mg. liver N per 100 g. rat
Vuj	6	106±2.3
Vuj minus glycine	6	112±1.8
Vuj minus arginine	7	120±3.5
Vuj minus histidine	7	110±2.8
Vuj minus isoleucine	7	106±3.8
Vuj minus leucine	7	112±1.6
Vuj minus lysine	7	104±2.3
Vuj minus methionine	7	98±1.9
Vuj minus phenylalanine	7	103±2.5
Vuj minus threonine	7	103±3.2
Vuj minus tryptophan	7	103±1.4
Vuj minus valine	7	106±2.2
Protein-free diet	14	89±1.8

tissues, sufficient amounts of most of the essential amino acids at least partially to correct deficient mixtures under the conditions of these experiments or that the liver has a limited capacity for synthesizing nutritionally incomplete proteins. The increase in liver nitrogen observed after feeding certain deficient amino acid mixtures may have been in part due to the higher concentrations of the remaining amino acids.

3. Acceptability of Amino Acid Diets in Short-Term Studies

The rejection of amino acid deficient diets by rats prompted us to investigate the time lapse between the rat's initial consumption of the diet and his rejection of it. Can he detect a deficient diet immediately or must he consume it for a period of time before its physiological imbalance becomes evident?

The pure amino acid mixture (Vuj$^+$)[5] used for these experiments was patterned after a VujN-type mixture which proved to be superior in liver repletion experiments. Adult rats, weighing an average of 260 g., were conditioned to eat 15 per cent casein diet one hour per day. They were then offered diets containing the amino acid mixture (15 per cent) or the same mixture with single amino acids omitted. Two other

[5] Per cent composition of Vuj$^+$ amino acid mixture: L-arginine, 6.35; L-histidine HCl-H_2O, 3.20; DL-isoleucine, 16.60; L-leucine, 18.70; L-lysine HCl, 9.80; DL-methionine, 7.05; DL-phenylalanine, 7.90; DL-threonine, 8.60; DL-tryptophan, 1.85; DL-valine, 11.90; glycine, 7.95.

groups of rats served as controls and received casein diet or protein-free diet. Food consumption was determined after 15, 30, 60, 90, 120, and 180 minutes. During the subsequent hour casein diet was offered to all rats. In this report some of the data are omitted for simplicity of presentation (Table VII).

TABLE VII

Food Consumption Effect of Omission of Single Amino Acids from the Diet
G. per Rat

Diet	No. of rats	Test diet			Casein diet
		0-15 min.	0-60 min.	0-180 min.	3 hrs.-4 hrs.
Casein	6	6.7±0.90	11.8±1.10	15.0±1.10	1.7±0.56
Protein-free	6	5.1±0.61	10.5±1.27	12.4±0.92	1.2±0.50
Vuj+	9	2.2±0.41	4.1±0.47	7.0±0.54	8.0±0.95
Vuj+ minus arginine	9	2.3±0.29	4.4±0.67	7.0±0.89	6.4±0.65
Vuj+ minus glycine	5	2.1±0.23	4.0±0.33	5.4±0.51	8.3±0.74
Vuj+ minus histidine	12	2.1±0.34	3.9±0.63	6.7±0.84	7.8±0.68
Vuj+ minus isoleucine	12	2.7±0.34	3.8±0.46	4.3±0.47	7.4±0.52
Vuj+ minus leucine	12	2.2±0.50	4.2±0.66	6.8±0.90	7.5±0.78
Vuj+ minus lysine	5	2.8±0.56	4.0±0.34	6.2±0.54	7.0±1.10
Vuj+ minus methionine	8	3.1±0.76	6.0±0.76	8.0±0.83	6.6±0.59
Vuj+ minus phenylalanine	5	2.7±0.02	5.2±0.01	6.6±0.54	7.8±0.86
Vuj+ minus threonine	5	1.4±0.35	3.9±0.67	7.3±0.73	7.7±1.20
Vuj+ minus tryptophan	6	2.3±0.35	4.8±0.42	7.1±0.39	6.2±1.14
Vuj+ minus valine	6	2.8±0.47	4.2±0.55	4.9±0.78	6.8±0.58

The following conclusions seem to be valid under these conditions:

(1) The diets containing amino acids were immediately less acceptable to the rat than casein diet or protein-free diet, whether or not all of the essential amino acids were present.

(2) Omission of methionine, threonine, tryptophan, or arginine had the least depressing effect on food intake whereas omission of isoleucine or valine depressed the food intake most.

(3) The intake of threonine-free diet was initially depressed but subsequently increased. On the other hand, isoleucine-free and valine-free diets were initially consumed as well as or better than the diet containing the complete mixture, but the food intake was almost negligible after the first hour.

(4) Those rats which had voluntarily restricted their intake during the test period had a tendency to consume more casein diet after the test.

In a second experiment, the rats were conditioned to eat their casein diet in 8 hours each day and were offered the above diets for 6 hours. The results confirmed conclusions 1, 3, and 4, and 2 was largely con-

firmed. The threonine, tryptophan, or methionine-free diets appeared to be the least objectionable of the deficient diets.

These experiments indicated that certain amino acids (such as isoleucine and valine) might be more appealing to the rat, and others (such as methionine) might be relatively unpleasant.

The most direct approach to this problem seemed to be incorporate individual amino acids in protein-free diet and offer the diets to normal, hungry rats for a short period of time. Thus, 250 g. rats which had been conditioned to consume casein diet one hour each day were offered diets containing one amino acid at a concentration of 15 per cent. Food consumption was determined after 15 minutes and 1 hour, and casein diet was supplied in the subsequent hour.

The experiment was carried out in two sections, with a casein control group in each (Table VIII).

TABLE VIII

Food Consumption Effect of Including Single Amino Acids (15%) in Protein-Free Diet
G./Rat (Av. of 9 or 10 Rats)

	Test Diet		Relative to	Casein diet
Diet	0-15 min.	0-60 min.	controls, %	1 hr.-2 hrs.
Casein	3.5±0.3	8.3±0.6	—	1.2±0.25
Protein-free	4.3±0.45	8.3±0.65	100	1.6±0.3
Vuj+	3.3±0.55	7.4±0.8	89	1.7±0.65
Arginine	2.2±0.2	5.9±0.5	71	2.3±0.55
Isoleucine	2.7±0.4	7.3±0.75	88	2.0±0.5
Leucine	4.0±0.45	6.5±0.7	78	1.8±0.5
Methionine	3.9±0.45	7.1±0.85	86	1.5±0.3
Tryptophan	3.7±0.3	7.0±0.5	84	0.9±0.6
Casein	4.0±0.45	9.3±0.55	—	2.6±0.5
Glycine	3.0±0.15	6.7±0.15	72	2.5±0.1
Histidine	1.5±0.25	4.7±0.3	51	4.1±0.75
Lysine	2.2±0.25	5.8±0.75	63	2.3±0.35
Phenylalanine	3.4±0.2	7.3±0.5	78	2.5±0.4
Threonine	3.4±0.5	8.8±0.7	95	2.2±0.5
Valine	4.0±0.1	9.1±0.25	98	1.4±0.25

The results may be summarized as follows:

(1) The rats consumed 10 per cent less Vuj+ diet than casein diet.

(2) The diets containing valine, threonine, isoleucine, and possibly methionine and tryptophan were as acceptable as the diet containing all amino acids.

(3) The diets consumed least readily were those containing histidine,

lysine, or arginine. It is probably more than coincidental that these are the 3 basic amino acids, all fed in the form of hydrochlorides.

(4) The intake of leucine and phenylalanine diets was intermediate.

(5) Again, there was a tendency for the animals which had restricted their intake of amino acid diets to compensate by taking more casein diet in the subsequent hour. This shows that they were capable of consuming more food but had voluntarily restricted their intake of the test diets.

These findings, combined with those of the preceding sections, clearly demonstrate that appeal to the animal is a critical factor in *ad libitum* experiments. Physiological effects upon the organism resulting from the ingestion of inadequate or imbalanced diets may be contributing factors to the loss of appetite after a time, but initially, factors other than nutritional value must be of major importance. In general, proteins may be consumed well initially, but if the protein is inadequate, food consumption would be expected to decrease later. Amino acid mixtures, on the other hand, appear to lack appeal, and may be shunned even though they are nutritionally adequate. In solution, these amino acid mixtures are obviously even more objectionable, and are consumed in minimal quantities. It may be concluded that initial acceptance of such diets by the rat depends upon physiological factors such as flavor.

4. Nitrogen Balance Studies

The nitrogen balance approach to the study of the biological value of proteins and amino acids (7, 8) has certain advantages. The method is relatively simple to perform, and can be economical of both time and materials. Since it requires less material than growth or repletion methods, amino acid mixtures which are not eaten well can easily be administered orally or parenterally. The latter route of administration is desirable for the evaluation of preparations for parenteral alimentation in man, a point which shall receive emphasis later.

In common with animal growth methods, the nitrogen balance approach measures overall response. At the present state of knowledge, this appears to be a desirable attribute. As the knowledge of nutrition advances, however, it may eventually be desirable to determine the effects of various proteins and amino acid mixtures upon specific body organs and tissues in order that their value may be fully utilized in therapy.

a. Adequacy of Proteins and Protein Hydrolysates. A number of proteins have been compared in 4-day nitrogen balance tests in dogs (13) by a procedure similar to that employed by Allison, Seeley, and Ferguson (14). The per cent retention of nitrogen in the 4-day test

period relative to preceding and subsequent control periods is reproduced in Table IX. In rat studies (Table IX), a test period of 1 day after a

TABLE IX

Retention of Protein Nitrogen by Rats and Dogs

	Rats (300 mg. N/kg.)		Dogs (120 mg. N/kg.)	
N-Source	No. of tests	Per cent N-retention	No. of tests	Per cent N-retention
Gelatin	3	23±8	8	31±5
Brewers' yeast	5	70±4	10	62±3
Soy meal	5	71±2	9	78±3
Beef liver	4	73±5	10	66±2
Casein	4	79±6	10	74±2
Egg white	4	104±8	9	94±3
Lactalbumin	4	95±4	—	(100)

1-week period of protein depletion was employed and the rats were fed sufficient test protein to supply 300 mg. N per kg. body weight.

The results from the two species were markedly similar. While egg white and lactalbumin were definitely superior to casein, gelatin was inferior in both animals. In these tests, brewers' yeast, soya meal and beef liver were of slightly less biological value than casein.

When 4 protein hydrolysates (A-D) of commercial origin were given to dogs orally (13), only minor differences were found in nitrogen retention (Table X). When the same solutions were administered intravenously, however, significant differences in utilization were detected, not only in nitrogen retention but also in excretion of amino acids and peptides. In rat experiments, after intraperitoneal injection of the same preparations, qualitatively similar results were obtained (Table XI). Analytical data which serve to characterize these hydrolysates have been published (13).

These data suggest that a short term intraperitoneal test of protein hydrolysates or amino acid mixtures may be feasible, with one condition. When peptides are present, an erroneous, high nitrogen retention value may be obtained, due apparently to a lag in the elimination of unavailable nitrogen of certain preparations. When such material is present it is necessary to extend the test period to at least 4 days, or to administer the test solution one or two days before initiating urine collections.

Since the application of the nitrogen balance approach appeared promising, it seemed desirable to conduct further studies, employing deficient mixtures of pure amino acids. The mixture employed was the Vuj mixture referred to previously.

TABLE X

Retention of Protein Hydrolysates by Two Dogs

Preparation	α-Amino N in urine, as % of α-amino N injected						*Total N retained, per cent* [c]
	Free		*Bound* [a]		*Total* [b]		
	After oral administration						
A	1.6	1.3	3.9	5.6	2.4	2.4	61±5.8
B	1.4	1.6	4.3	6.6	2.2	3.1	64±4.8
C	2.6	2.7	2.6	1.5	2.6	2.1	66±2.0
D	4.4	4.2	12.6	8.3	6.5	5.5	67±3.9
E	1.4	0.3			1.0	0.1	73±9.1
F	0.4	0.1			0.8	0.0	61±4.0
	After intravenous infusion						
A	12.5	10.7	31.1	29.9	19.5	17.9	45±4.0
B	21.9	18.9	24.3	11.3	22.6	16.6	55±1.0
C	12.9	11.0	46.0	44.6	27.8	25.7	0±13.0
D	8.5	12.3	32.3	19.0	14.4	14.5	40±1.0
E	7.5	3.0			8.8	3.2	57±5.0
F	6.6	7.1			8.1	8.2	43±6.0

[a] α-amino N after hydrolysis minus α-amino N before hydrolysis.

[b] α-amino N after hydrolysis.

[c] Relative to lactalbumin as 100 per cent; averages of 3 or 4 figures. Labco casein gave values of 70, 72, 73, 76, 76, 76 and 78 per cent.

TABLE XI

Retention of Protein Hydrolysates by Rats
(200 mg. N/kg./day, I.P.)

N-Source [a]	No. of rats	α-Amino N in urine, as per cent of α-amino N injected [b]			Total N retained, per cent	
		Free	*Bound*	*Total*	*1st 2 days*	*2nd 2 days*
A	6	6.7	25.4	13.8	40±9	46±6
B	6	4.1	20.2	8.4	48±5	55±7
C	6	6.5	35.5	19.5	50±6	34±3
D	6	2.3	19.3	6.7	43±5	59±7
E	6	0.5	—	0.8	60±4	67±5
G	4	—	13.5	13.5	29±4	14±3

[a] A-D Protein hydrolysates of commercial origin.
E Amino acid mixture, free of peptides.
G Gelatin.

[b] α-Amino nitrogen excretion determined on pooled urines.

b. Nitrogen Retention After Feeding Deficient Amino Acid Mixtures and the Effect of Supplying the Deficient Amino Acid Previously. When single amino acids were omitted from the mixture fed orally to supply a total of 300 mg. N per kg. in one day, nitrogen retention was least influenced by the omission of arginine, histidine, leucine, or lysine and most depressed by the omission of methionine, valine, or isoleucine. The results (Table XII) are in general agreement with those of Bur-

TABLE XII

Per Cent Retention of N by Rats Fed Deficient Amino Acid Mixtures and Effect of Supplying the Missing Amino Acid Previously

(300 mg. N/kg. orally, one day)

Missing amino acid	Unsupplemented	Supplemented	
		2 hrs.	*4* hrs.
None	89±2	—	—
Methionine	14±5	78±7	77±4
Valine	34±4	73±6	66±4
Isoleucine	41±8	49±3	64±3
Phenylalanine	53±5	74±2	77±2
Threonine	53±6	91±8	91±5
Tryptophan	50±8	86±5	88±5
Leucine	70±4	75±5	70±4
Arginine	78±4	—	—
Histidine	83±4	—	—
Lysine	68±3	—	—

roughs, Burroughs, and Mitchell (15), whose experiments were of several weeks duration. When the missing amino acid was administered to the rats 2 or 4 hours before each of two portions of the deficient mixture, improvement in nitrogen retention was noted in almost all groups. These results demonstrate that, under these conditions, a significant portion of single amino acid feedings remains in the body of the rat 2 to 4 hours. Hier (16) has shown in rats that after the administration of single amino acids, their concentrations in the blood may remain elevated for 1 to 12 hours. Upon the administration of a "complete" amino acid mixture, the disappearance of all the amino acids from the blood would conceivably be more rapid due to utilization of the mixture. On the other hand, single amino acids should be little utilized, and disappearance from the blood may depend almost entirely upon rates of excretion and deamination.

Previous investigations (17, 18, 19, 20) have given rise to the concept that all amino acids must be applied simultaneously for optimal utiliza-

tion. However, the evidence deals primarily with the delayed supplementation of tryptophan-free casein hydrolysates. In one report (20), two diets, each containing 5 of the "essential" amino acids, were offered to rats in alternating periods. The rats did not utilize the two diets as well as a diet containing all 10 amino acids. However, the consumption of amino acid deficient diets has been shown (21) to be seriously limited and the two incomplete diets were not utilized even when both were available to the rat at the same time. In the present experiments, evidence of utilization of *single* "essential" amino acids administered 2 or 4 hours *prior to* administration of deficient mixtures has been obtained. It appears that more definitive data are needed to establish or qualify the above concept of the essentiality of simultaneous feeding of amino acids.

c. Nitrogen Retention After Intraperitoneal Administration of Deficient Amino Acid Mixtures. The effect of deleting single amino acids from the mixture before injecting intraperitoneally into rats had similar effects on nitrogen balance in 3 to 6-day experiments (Table XIII). The essentiality of the individual amino acids, as found after

TABLE XIII

Per Cent Retention of N After Intraperitoneal Administration of Deficient Amino Acid Mixtures to Rats

Missing amino acid	300 mg. N/kg./day	200 mg. N/kg./day		
	3 days	*1st 2 days*	*2nd 2 days*	*3rd 2 days*
None	74±4	70±2	69±6	70±5
Glycine	74±3	—	—	—
Arginine	62±7	—	—	—
Histidine	62±3	—	—	—
Isoleucine	21±2	37±3	46±2	45±3
Leucine	44±3	48±6	51±2	54±3
Lysine	62±5	—	—	—
Methionine	9±6	11±3	11±2	26±3
Phenylalanine	35±0	48±4	50±2	57±2
Threonine	40±2	45±6	30±3	32±2
Tryptophan	41±4	45±4	51±2	60±2
Valine	25±9	24±3	39±3	47±3

oral administration, was confirmed. In the 6-day experiment there were indications that omission of tryptophan or valine became less critical as the experiment progressed, and that omission of threonine became more critical with time. It appeared that the labile proteins available for internal supplementation at the beginning of the experiment con-

tained insufficient amounts of tryptophan and valine but after 4 days, the available tissue protein contained more of these two amino acids. It seems unlikely that the tissues acquired a greater capacity for synthesizing these two amino acids as the experiment progressed.

d. Imbalance Effect on Nitrogen Retention. Adverse effects of excesses of single amino acids have been reported to occur when an amino acid, particularly methionine, was added to a low protein diet (22, 23). It seemed appropriate to determine whether all amino acids had adverse effects when supplied in excess in amino acid mixtures. Groups of 3 rats were fed protein-free diet for one week and were then dosed orally with the Vuj mixture (300 mg. N per kg.) and excesses of individual amino acids (100 mg. N per kg.). In these studies (Table XIV), only methionine had an imbalance effect, since for each mg. of excess methionine nitrogen administered, 1.86 mg. of nitrogen was excreted in the urine. The nitrogen supplied as excess tryptophan or lysine was quantitatively lost in the urine, but no significant imbalance effect was noted. Excess aspartic acid nitrogen was utilized as well as excess Vuj, with only 26 per cent lost in the urine. Roth and Allison (22) have found that when 4.8 per cent glycine or methionine is added to a 12 per cent casein diet, the excess amino acid nitrogen is lost in the urine but part of excess arginine nitrogen, similarly fed, is retained by the animal.

TABLE XIV

Effect of Imbalance in Amino Acid Mixture Composition upon N Excretion by Rats
(Oral dose, VUJ, 300 mg. N/kg., plus single amino acid, 100 mg. N/kg., one day)

Amino acid in excess	No. of groups	Per cent N retained	Per cent excess N excreted
None	17	78±1	—
Methionine	6	37±3	186
Tryptophan	6	56±4	110
Lysine	6	57±4	106
Leucine	8	62±3	86
Arginine	6	65±1	74
Phenylalanine	7	67±4	66
Isoleucine	9	71±4	50
Threonine	9	73±3	42
Histidine	6	74±3	38
Glycine	3	73±3	42
Aspartic Acid	3	77±4	26
Vuj	3	76±1	30

In the experiments reported here, glycine, arginine and histidine were partially utilized when given in excess, and mixtures deficient in

any one of these amino acids were well utilized. Tryptophan, lysine and leucine were apparently not harmful when given in excess, neither were they utilized in excess.

Since either a deficit or an excess of methionine leads to increased nitrogen excretion, it is obvious that the level of this amino acid is of critical importance. Miller (24) has shown that the feeding of methionine to animals on a protein-free diet spared body nitrogen, and although the data of Roth and Allison (22) did not reveal an imbalance effect of excess methionine on nitrogen excretion, these authors and Kade and Sheperd (23) reported a loss of body weight in rats fed excess methionine in low protein diets.

III. Discussion

Protein hydrolysates and amino acid mixtures have been tested biologically by a number of methods, yielding a variety of results. The values obtained apply to the specific conditions employed in each experiment, and in the absence of correlated studies, the conclusions drawn from one type of study do not tell us how a given preparation will perform under other conditions. This is particularly true of preparations designed for parenteral feeding of patients, since oral feeding is often the method of convenience in the laboratory. However, if a laboratory test of "biological value" is desired, it seems logical to employ conditions that would come as close as possible to measuring the properties of the product that are necessary for its clinical application. Our experience has led us to believe that a parenteral nitrogen balance test in well-controlled dog or rat experiments is the best available solution to this problem. Since dog experiments are expensive and impractical, a rat intraperitoneal test appears to be the least undesirable biological method at the present time.

The recently announced data of Rose (25) regarding the oral amino acid requirements of normal men introduces another approach which probably comes closer to the true solution than any animal test. In the absence of data to the contrary, one must assume that the requirements of the average patient are qualitatively similar and quantitatively either the same or increased. Thus, standards for the oral feeding of amino acid preparations could be conceived relatively easily, by providing that the daily intake contain at least as much of each amino acid as Rose found necessary for maintenance of nitrogen balance in adults.

For a parenteral product, then, one would merely need to add a margin of safety to provide for less efficient utilization by the parenteral route. It remains to be determined how much the patient's requirements

differ from the normal. If such information were available, it might result in the tailoring of amino acid mixtures to suit the type of patient, but a great deal of information would be needed to accomplish this.

The presence of peptides in a parenteral product seriously complicates any proposal to employ amino acid analyses in the control of such preparations. If "unavailable" peptides are present, the assays should be carried out on the product as it is supplied and provision should be made to avoid including these peptides in the assays. On the other hand, if peptides that are "available" after parenteral administration are present, they should be included in the analyses, so complete hydrolysis of the product prior to assay would be indicated. If a preparation contains both "available" and "unavailable" peptides, it is difficult to visualize any way of applying amino acid analyses to its control. Both types of peptides have been reported (26, 27). In such instances, a biological method, such as an intraperitoneal rat test, would appear to offer a reasonable solution.

There is no question regarding the desirability of determining what combinations of amino acids or other factors tend to favor the synthesis of one tissue protein over another. The clinical implications are obvious, not only for parenteral feeding but oral feeding as well. When limited amounts of protein or amino acids can be ingested or infused, it would be highly desirable to supply that protein or amino acid mixture which would be most efficient for that particular patient.

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Dietary Proteins and Synthesis of Tissue Proteins

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CONTENTS

I. Introduction 94
II. The Effect of Dietary Proteins on the Synthesis of Plasma Proteins . . 96
1. Protein Depletion on Plasma Proteins 96
2. Repletion with Different Dietary Proteins 99
III. The Effect of Dietary Proteins on Repletion of Liver Proteins 107
IV. The Role of Dietary Proteins in the Hypertrophy of Gonads 108
V. Discussion 111
References 113

I. Introduction

The body of an animal contains the following primary constituents: water, inorganic salts, lipids, proteins, and carbohydrates. Among these classes of compounds, proteins may be considered as the most important because they perform a great number of physiological functions. For example, all pituitary hormones from the "master gland" which controls the secretion of most of the other important endocrine glands, are proteins. Most enzymes and antibodies are likewise proteins. During the life processes, these protein molecules are constantly destroyed and must be replaced by synthesis from amino acids or peptides derived from food. In order to utilize these amino acids, it is necessary that certain of the so-called essential ones be made available to the body at the appropriate time. The works of Rose (1), Whipple (2), and Cannon (3) demonstrated that among numerous amino acids isolated from proteins, only 10 of them are essential to animals, like dogs or rats, for the promotion of growth, for the maintenance of nitrogen balance, and for the regeneration of plasma proteins. These amino acids are considered essential

[1] The data presented in this paper were to a large extent collected at the Squibb Institute for Medical Research, New Brunswick, New Jersey. The author is appreciative of the collaborative efforts of all his former associates, particularly Dr. C. Alper, Misses S. Quaid, L. Hall, and L. Thomson. The work reported in Tables IX, X, and XI was supported by the grant-in-aid from the Squibb Institute for Medical Research and from Hoffmann-LaRoche, Inc.

because the animals are not able to synthesize them in the body from the material in the diet at a speed, commensurate with the demands for normal growth, and the lack of any one of them will result in impaired growth, in considerable loss of body weight, and ultimately in death. Such a definition, as recognized by Rose and his coworkers (4), may not include other important physiological functions such as reproduction, lactation, or detoxication. For example, lysine is essential for growth of young rats, but according to Mitchell (5) this amino acid is not needed for the maintenance of nitrogen equilibrium in the normal, sexually mature rats. On the other hand, lysine is essential for the maintenance of nitrogen equilibrium in the dog (6) and for adequate human nutrition (7). It was suggested by Mitchell (5) and Borman *et al.* (4) that unless synthesis of a given amino acid can keep pace with the requirement, be it for growth or maintenance, deficiency will soon manifest itself. Albanese (8) has also emphasized that the essentiality of an amino acid may not be indicated by nitrogen balance measurements.

It may also be pointed out that different species of animals vary in the quantitative requirements of an amino acid. The essentiality of only 7 amino acids for man was suggested, while 9 amino acids were believed to be indispensable to rats. Recently Cox and his associates (9) demonstrated that the addition of cystine or methionine to an enzymatic digest of casein increases the rate of growth of rats and that the addition of methionine increases nitrogen retention when the casein hydrolysate is administered to dogs intravenously. However, the addition of methionine does not affect the nutritive value of the casein hydrolysate, when fed to man. They (9, 10) further demonstrated that although lactalbumin is superior to casein for growth of rats and for the maintenance of nitrogen equilibrium in rats as well as in dogs, these two milk proteins are equivalent in the support of nitrogen balance in man.

Elman (11) demonstrated that the injection of tryptophane to dogs fed intravenously an acid hydrolyzed casein lacking in this essential amino acid brought about retention of the hydrolysate nitrogen, provided the supply of tryptophane was not unduly delayed. The studies of Harte (12) and Geiger (13) provided additional evidence that "incomplete" amino acid mixtures are not stored in the body and that the presence of all the essential amino acids at appropriate time is necessary for the utilization of the dietary amino acids.

From the important contributions of these investigations it can be concluded that the presence of all the essential amino acids in a dietary protein is a prerequisite for adequate nutrition. On the other hand, if adequate supply of all the essential amino acids at the proper site of the body and at the appropriate time is all that is required, then the defi-

ciency of poor proteins low in the content of certain essential amino acids, can be made up by an increase of dietary intake or by supplementation of the deficient amino acid. This is not in accordance with experimental facts. Recent studies of Woolley (14) and Womack and Rose (15) suggest that in addition to certain essential amino acids, some unknown nutritional factor is needed for optional growth. The growth experiments necessary to substantiate such a hypothesis are complicated by the synthesis of yet unidentified vitamins by the bacterial organisms in our intestinal flora.

In this communication, data will be presented to demonstrate that the synthesis of tissue proteins depends not only on the presence of the essential amino acids in adequate amounts but also on the dietary proteins from which the supply of these building stones and possibly of some nutritional factors is drawn.

II. The Effect of Dietary Proteins on the Synthesis of Plasma Proteins

1. Protein Depletion of Plasma Proteins

Chow, Allison, and others (16, 17) studied the effects of protein depletion on the plasma proteins of dogs. Protein depletion was achieved by offering a group of well-fed normal dogs a protein-free diet containing sufficient calories and vitamins to adequately meet the daily requirements. After 6 to 8 weeks of such a diet, the plasma protein concentration of these animals dropped from 6.5–7.0 to 4.0–4.5 g. per 100 ml. The plasma volume likewise decreased about 20 per cent. Electrophoretic analysis of plasma of dogs taken before and after depletion demonstrate that protein depletion resulted in a decrease of per cent of albumin and globulins except the alpha globulins whose percentage was markedly increased. These results are summarized in Table I. In this table are included two other methods of protein depletion. One was to restrict the caloric intake by offering the animals a diet containing adequate percentage of proteins, carbohydrate, fat, minerals, and vitamins, only to the extent of 30 cal. per kg. body weight per day. Depletion of protein reserves with such a procedure took place rapidly. In 2 to 3 weeks the plasma protein concentration decreased to about 4.5 g. per cent. The third method of protein depletion was achieved by daily plasmapheresis for 10 days while the animals were on a protein-free diet.

It is interesting to note that the same results ultimately occurred regardless of the method of depletion, namely, by feeding a protein-free diet, with or without plasmapheresis, or by feeding a diet adequate in

TABLE I

Composition of Plasma Protein of Dogs Before and After Protein Depletion by Different Methods

Method of depletion	No. of dogs used	Treatment	Plasma vol., ml.	*Plasma Proteins*				Total circulating *plasma protcins*			
					Fractions						
					Albumin,	*Globulins*		Albumin,	*Globulins*		
				Total, g. %	%	$^{a}1 + {}^{a}2$, %	γ, %	g.	$^{a}1 + {}^{a}2$, g.	γ, g.	A/G
A [a]	15	Control	473	6.21	42	16	9	12.4	4.5	2.5	0.75
	15	Depleted	427	4.48	21	26	9	4.0	4.9	1.8	0.27
A [b]	15	Control	603	6.74	40	16	8	16.0	6.6	3.0	0.67
	15	Depleted	510	5.04	21	26	11	5.3	6.8	3.0	0.27
A [c]	5	Control	500	6.37	40	15	9	12.7	4.8	2.9	0.67
	5	Depleted	425	4.52	21	25	10	3.8	4.5	1.6	0.27

[a] By plasmapheresis and protein-free feeding.
[b] By protein-free feeding alone.
[c] By feeding a diet low in calories.

proteins but insufficient in caloric intake. The decrease of per cent of albumin reached a plateau of approximately 20 per cent, with albumin to globulin ratio of 0.25. This ratio could be lowered only by further prolonged protein deprivation, resulting in a decrease of the alpha globulins. Feeding of casein or lactalbumin or the hydrolysate of either protein at this junction failed to bring the animals into nitrogen equilibrium regardless of the quantity of intake (within 0.20–1.0 g. nitrogen per kg. body weight per day), unless 15 units of liver extract were given simultaneously for at least 5 days. The effect of liver extract on the ability of severely protein depleted animals to assimilate an otherwise adequate casein previously digested with purified trypsin is shown in Table II.

Before the discussion on the possible effect of dietary proteins, it is appropriate that the importance of degree of depletion of protein reserves and of the mode of administration be discussed, since they likewise play a role in the synthesis of tissue proteins. Alper, Chow, and DeBiase (18) studied the utilization of enzymatic digest of casein by dogs depleted of protein reserves either by protein-free feeding alone or in conjunction with plasmapheresis. During the first part of the experiment, the pyrogen-free hydrolysate containing 40 per cent of its total nitrogen as amino nitrogen was fed parenterally for a prolonged period (4 weeks) at a level of high caloric intake (supplied orally in the form of protein-

TABLE II

The Effect of Intramuscular Injection of Liver Extract on the Nitrogen Retention by Dogs Severely Depleted of Protein Reserves

Group	No. of dogs	Weeks on protein-free diet	Albumin Globulin	Days of feeding	Nitrogen [a] intake	Nitrogen [a] output	Nitrogen [a] balance
A	3	18	0.12	10	300	380	— 80
B	3	20	0.14	10	1000	1100	— 100
C	3	18	0.14	10	300	160	+ 140

All animals were given 60 cal. per kg. body weight per day. 1.0 ml. of 15 unit liver extract was given daily for first 5 days to animals in Group C.

[a] These figures are averages expressed as mg. per kg. body weight per day.

free diet at 70 to 90 cal. per kg. body weight per day) and of high protein intake (0.35 g. of hydrolysate nitrogen per kg. body weight per day), to insure an adequate and surplus amount of nutrients. Subsequently, these same animals received orally for an additional period of like number of weeks of the same hydrolysate dried by lyophilization, to ascertain nitrogen retention and plasma protein regeneration during both phases of the experiment.

Their results on the nitrogen balance experiments (see Table III) demonstrate two interesting points. (1) Dogs depleted of protein reserves by the protein-free feeding procedure (Group 1) were in more positive nitrogen balance than those depleted by plasmapheresis (Group 2), when the hydrolysate was given intravenously. During this period, animals in both groups regenerated plasma proteins. (2) When administration was changed from intravenous to oral route, all dogs were in greater nitrogen balance than in the previous period.[2]

Their data led them to draw the following conclusions: (1) In hypoproteinemia affected by restricted plasmapheresis, the "reserve protein stores" of the experimental animals are not permanently depleted although plasma protein concentration and volume are markedly reduced. On the other hand, feeding an experimental animal a protein-free diet over a long period of time will drain the "reserve protein stores" first and finally deplete the plasma proteins. Animals depleted by the latter method, where there is extensive loss of tissue proteins, might be expected to show different utilization of the parenteral nitrogen supplement than the animal not so depleted. (2) The discrepancy of extremely high

[2] For an interesting discussion on the differences between oral and parenteral administration, the readers are referred to p. 196 of "Parenteral Alimentation in Surgery" by R. Elman, published by P. B. Hoeber, Inc., New York, 1947.

retention of orally administered nitrogen after partial repletion by parenterally administered nitrogen points to the importance to the pattern of amino acids and peptides offered to the animal for the synthesis of its body tissues.

TABLE III

The Effect of Route of Administration of Casein Hydrolysate on Nitrogen Balance and Plasma Protein Regeneration of Dogs

		Period I (*4 weeks of I.V. feeding*)		Period II (*4 weeks of oral feeding*)	
Group no.	No. of dogs	nitrogen balance [A]	gain in plasma protein [B]	nitrogen balance [A]	gain in plasma protein [B]
1	6	+ 0.071 ± 0.023	6.6 g.	+ 0.165 ± 0.027	6.3 g.
2	4	+ 0.023 ± 0.018	1.8 g.	+ 0.145 ± 0.015	3.5 g.

A = Average nitrogen balance expressed as g. of nitrogen per kg. body weight per day with standard error.

B = Gain in total circulating plasma proteins in g. The gain during the second period represents the additional increase of total circulating plasma proteins following the oral administration.

Group 1 = Dogs depleted of proteins by protein-free feeding for 6-8 weeks.

Group 2 = Dogs depleted of proteins by restricted plasmapheresis.

2. Repletion with Different Dietary Proteins

The fact that various food proteins differ qualitatively in their ability to promote synthesis of plasma proteins has been suggested by investigators such as Whipple (19), Melnick (20, 21), Weech (22, 23, 24), and Cox (25), and their associates. By means of the plasmapheresis technique, Whipple and his collaborators demonstrated that some dietary proteins may favor the production of plasma albumin and others may favor the production of plasma globulins. The limitations of these studies were the inherent inadequacy of the chemical methods for the determination of albumin and globulins content and the lack of information of plasma volume in some reports. With the advance of the electrophoretic analyses for the determination of plasma protein components and with the improvement in the accuracy and reliability in the determination of plasma volume, we were, therefore, interested in reexamining the question whether various common dietary proteins (such as egg white, whole egg, lactalbumin, casein, and wheat gluten) of different nutritive values as measured by growth or nitrogen balance tests differ in their ability to regenerate plasma proteins. The availability of protein-depleted dogs, as described above, is valuable in such

TABLE IV

Effects of Oral Feeding of Various Proteins on Total Albumin and Globulins of Dogs Depleted in Proteins [a]

Protein fed	No. dogs used	Total circulating protein after feeding for [b]		Total circulating albumin after feeding for [b]		Total circulating globulins after feeding for [b]		A/G after feeding for		
		2 wks.	4 wks.	2 wks.	4 wks.	2 wks.	4 wks.	0 wks.	2 wks.	4 wks.
Egg white	10	117 ± 5	132 ± 17	117 ± 8	133 ± 12	118 ± 5	126 ± 16	0.28	0.27	0.26
Lactalbumin	5	115 ± 10	128 ± 10	185 ± 21	252 ± 23	100 ± 9	100 ± 9	0.22	0.42	0.58
Whole egg	5	148 ± 7	154 ± 7	206 ± 21	289 ± 22	138 ± 6	129 ± 7	0.19	0.28	0.43
Casein	4	138 ± 9	141 ± 5	177 ± 17	216 ± 19	130 ± 8	124 ± 4	0.25	0.33	0.39
Wheat gluten	5	135 ± 4	135 ± 4	162 ± 6	168 ± 7	130 ± 5	129 ± 5	0.17	0.21	0.22
Lactalbumin hydrolysate	6	141 ± 8	145 ± 6	230 ± 5	362 ± 8	115 ± 6	98 ± 7	0.21	0.44	0.85
Casein hydrolysate	5	138 ± 7	150 ± 3	223 ± 4	238 ± 8	119 ± 11	124 ± 10	0.22	0.41	0.51

[a] All the proteins were given at a level of 0.35 g. N/kg. body weight/day except wheat gluten, which was given at a 0.6 g. level.

[b] The total circulating proteins before feeding are taken as 100 %. The figures after the ± signs are standard errors.

a study since they are rather reproducible. To this end (Chow, Alper, DeBiase, 26, 27), several groups of protein-depleted animals were fed diets containing one of the 5 test proteins so that each animal received 0.35 g. of nitrogen and 80 cal. per kg. body weight per day for a period of at least 4 to 6 weeks. The exception was that 0.6 g. of wheat gluten nitrogen was given instead of 0.35 g. because of its low biological value. Determinations of total circulating plasma proteins as well as albumin and globulins were made before feeding and again 2 and 4 weeks after feeding.

Our data (Table IV) demonstrate that supplementation of the protein-free diet with any one of the 5 proteins or 2 hydrolysates stimulated an increase in the total circulating plasma proteins. The difference in the per cent increases was not very marked among the test substances even though the nutritive values, as measured in terms of growth efficiency, differed by several fold. Furthermore, there appeared to be a lack of correlation between nitrogen balance index and plasma protein regeneration properties. For example, lactalbumin with a nitrogen balance index of unity did not stimulate as effectively the synthesis of plasma proteins as casein or whole egg proteins with indexes of 0.80 and 0.95 respectively. Egg white which has the highest biological value among the five proteins failed to bring about regeneration of plasma proteins to the same extent as casein even when feeding was continued for 8 weeks. A comparison of the whole proteins and their respective hydrolysates showed that the plasma protein regeneration properties of casein was not improved by tryptic digestion, while the tryptic digest of lactalbumin was definitely superior to the whole protein. This might be explained by the fact that casein is rapidly hydrolyzed by the proteolytic enzymes normally present in the digestive tracts of the animals, whereas lactalbumin is not.

Since hypoproteinemia is always accompanied by a severe loss in the total circulating albumin, it seems reasonable to expect that repletion with any one of the dietary proteins, should regenerate the albumin fraction in preference to other plasma proteins. However, our data demonstrated that the 5 test proteins varied in the effectiveness to stimulate the production of albumin. For example, the increase was 230 per cent for lactalbumin hydrolysate and 117 per cent for egg white proteins after two weeks of feeding. The latter was surprisingly ineffective for the purpose of plasma albumin synthesis. The rates of regeneration of albumin by the hydrolysates were again significantly greater than those of the corresponding proteins (185 per cent against 230 per cent for lactalbumin and its hydrolysate, 177 per cent against 233 per cent for casein and its hydrolysates, respectively).

A careful examination of data on the globulin regeneration shows that while 4 of the proteins administered stimulated the production of globulins in varying degrees, dogs fed with lactalbumin or its hydrolysates failed to gain in globulins. This difference was also reflected in the albumin to globulin ratios, being highest for the last mentioned protein.

The changes in albumin-globulin ratio (see Table V) reflect the difference between the casein and lactalbumin hydrolysates. The control ratio

TABLE V

The Average Total Circulating Albumin, α-, β- and γ-Globulins in g./m.² of Body Surface and Albumin-Globulin Ratios in the Control, Depleted and Repleted Condition on 5 Dogs Repleted with a Casein Hydrolysate and 6 Dogs Repleted with a Lactalbumin Hydrolysate [a]

Condition	Circulating albumin, g./m.²	Circulating globulin			A/G
		Alpha, g./m.²	Gamma, g./m.²	Other, g./m.²	
		Casein Hydrolysate			
Control	25.7	9.7	4.8	19.6	0.75
Depleted	7.6	10.1	3.9	20.2	0.22
Repleted	23.4	10.9	6.0	28.7	0.51
		Lactalbumin Hydrolysate			
Control	28.4	8.1	6.0	21.5	0.80
Depleted	6.9	8.0	3.8	21.4	0.21
Repleted	29.5	7.3	5.3	22.3	0.85

[a] Each dog received 0.35 g. of hydrolysate nitrogen/kg. of body weight/day for 30 days.

in the dogs which were to be repleted with casein hydrolysates was 0.75; during depletion it decreased to 0.22, and after repletion the ratio returned only to 0.51 due to high globulin values. The control ratio of dogs to be depleted with lactalbumin hydrolysate was 0.80; during depletion it decreased to 0.21 and after repletion the ratio increased to 0.85.

Our first attempt (Bolling, Block, and Chow, 28) to explain the difference was based on the possible difference in the amino acid patterns of our casein and lactalbumin hydrolysates and perhaps the similarity of amino acid contents between lactalbumin and dog plasma albumin. Therefore, analyses for the essential amino acids of the two hydrolysates as well as of the electrophoretically homogeneous albumin and of the total globulins of dog plasma were performed. Our results demonstrate that the two hydrolysates differed most significantly in their

ratios of cystine to methionine. Since, of the nitrogen retained, less than 5 per cent was utilized for the synthesis of plasma proteins, it is unlikely that the inability of dogs fed with lactalbumin hydrolysate to induce the production of globulins was due to any limiting factor of any amino acids fed. Furthermore, the analyses of albumin and globulins did not reveal any specific requirements of certain amino acids which the hydrolysates had to supply for the synthesis of the two fractions of plasma proteins.

The superiority of lactalbumin over casein in promoting the growth (29) of young rats or in supporting nitrogen balance in dogs (30) has been well demonstrated, and the difference can be compensated for by feeding the animals a higher level of casein. It is, therefore, of interest to ascertain whether the production of specific protein components in plasma is similarly dependent on the amount of absorbed nitrogen, which might account for the difference in the plasma protein regeneration properties of these two milk proteins. To this end, studies (27) were undertaken which involved feeding a group of protein-depleted dogs with a casein hydrolysate at a nitrogen level sufficient to stimulate a rapid and pronounced regeneration of plasma proteins. For comparison, three other groups of protein-depleted animals were fed 3 different levels of a tryptic digest of lactalbumin, so that the retained nitrogen was either greater, approximately equal to, or less than that retained by dogs receiving the casein hydrolysate. In addition, two other groups of dogs were fed the same level of casein hydrolysate as the first group but were given 1 per cent or 2 per cent methionine as supplement in order to increase the nitrogen retention to approximately that of the animals receiving the highest dosage of lactalbumin hydrolysate. The regeneration of various protein components as well as the nitrogen retention were measured for the whole period of feeding.

The results (Table VI) demonstrate that all 6 diets were adequate in supporting nitrogen balance (see column 4) during the period of repletion. At the level of nitrogen intake of 0.35 g. per kg. body weight per day, the lactalbumin hydrolysate was more effective than casein hydrolysate which supplemented the addition of 1 per cent or 2 per cent methionine. Thus at 0.35 g. nitrogen level, half of the casein hydrolysate nitrogen was retained and the retention was increased to 70 per cent by the addition of 1 per cent or 2 per cent methionine. On the other hand, at least 80 per cent of the lactalbumin hydrolysate nitrogen was retained.

The results (see Table VII) show that dogs fed with any one of the 3 levels of lactalbumin hydrolysate regenerated albumin and no globulins. Those fed casein hydrolysate, however, produced both albumin and globulins irrespective of supplementation of methionine.

The data presented above lead us to believe that the difference in the plasma protein regeneration properties of casein and lactalbumin is neither due to the amino acid composition nor to the amount of amino acids retained by the animal. It, therefore, appears plausible that some nutritional factors other than amino acids play a role in directing the synthesis of plasma proteins. To this end we have fractionated a casein hydrolysate solution with alcohol and found that the addition of 1 per cent of the fraction, soluble in 60 per cent but insoluble in 80 per cent

TABLE VI

The Levels of Absorbed Nitrogen and the Plasma Protein Regeneration

Protein supplement	No. dogs used	Level of intake [a]	Nitrogen balance [b]	Retained "biological value" [c]	Plasma protein nitrogen, [d] %
Lactalbumin hydrolysate	5	0.35	+ 0.186	0.8	4.6
Lactalbumin hydrolysate	4	0.25	+ 0.118	0.8	3.0
Lactalbumin hydrolysate	4	0.15	+ 0.072	1.0	2.7
Casein hydrolysate	5	0.35	+ 0.117	0.5	4.5
Casein hydrolysate + 1 per cent methionine	3	0.35	+ 0.175	0.7	4.5
Casein hydrolysate + 2 per cent methionine	3	0.35	+ 0.165	0.7	5.0

[a] Level of intake is expressed as g. nitrogen per kg. body weight per day.

[b] Nitrogen balance is the difference between nitrogen intake and nitrogen excretion, expressed in g. nitrogen per kg. body weight per day.

[c] "Biological value" is calculated according to the equation of Allison (30): (NB = (BV) (AN) — (EN). (See text.)

[d] Per cent of the total absorbed nitrogen used for the synthesis of total plasma protein.

alcohol, to a lactalbumin hydrolysate improved its globulin regeneration properties (see Table VIII). It is hardly conceivable that supplementation to the extent of 1 per cent was enough to sufficiently alter the amino acid pattern of the lactalbumin hydrolysate. These data, therefore, demonstrate the existence of a fraction in casein hydrolysate which was effective in globulin regeneration when added to lactalbumin hydrolysate in a small quantity.

Some of the chemical and biological characteristics of this fraction were also determined (see Table IX). Chemical analyses show that it contained 14.6 per cent total nitrogen by weight. Sixty-eight per cent of the total nitrogen was amino nitrogen according to the ninhydrin method. Microbiological assay for Woolley's strepogenin factor using

TABLE VII

The Effect of Oral Feeding of Casein or Lactalbumin Hydrolysates at Different Levels of Nitrogen Intake on Total Circulating Plasma Proteins of Dogs Depleted in Proteins

Level of feeding	No. dogs	Total circulating proteins [a]		Total circulating albumin [a]		Total circulating globulins [a]		A/G	
		3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks	0 weeks	6 weeks
Lactalbumin hydrolysate: 0.35 g. N/kg	4	115 ± 10.0	128 ± 10.0	185 ± 21	252 ± 23	100 ± 8.6	100 ± 9.3	0.22	0.60
Lactalbumin hydrolysate: 0.25 g. N/kg	4	121 ± 7.1	137 ± 8.7	193 ± 21	251 ± 40	106 ± 5.2	105 ± 6.5	0.23	0.46
Lactalbumin hydrolysate: 0.15 g. N/kg	4	108 ± 9.3	123 ± 7.1	176 ± 22	227 ± 16	97 ± 9.6	105 ± 7.0	0.16	0.35
Casein hydrolysate: (0.35 g. N/kg) plus 1 per cent methionine	4	124 ± 6.1	141 ± 9.0	172 ± 23	245 ± 35	114 ± 5.7	120 ± 3.0	0.23	0.41
Casein hydrolysate: (0.35 g. N/kg) plus 2 per cent methionine	3	133 ± 7.9	146 ± 6.1	196 ± 18	262 ± 29	116 ± 4.8	118 ± 1.0	0.26	0.44
Casein hydrolysate: (0.35 g. N/kg) alone	5	138 ± 7.1	150 ± 2.8	195 ± 7.5	210 ± 10	113 ± 10.6	136 ± 9.1	0.22	0.51

[a] Calculated as per cent of the amount determined prior to protein feeding, with standard error.

L. caseii gave negative results. This is interesting since the original casein hydrolysate contained 5 units per g. and the lactalbumin hydrolysate contained about one unit per g. Microbiological assay for crystal-

TABLE VIII

The Effect of Addition of a Fraction of Casein Hydrolysate to Lactalbumin Hydrolysate on Plasma Protein Regeneration

Level of feeding	No. of dogs	TCA [a]	TCG [b]
0.35 g. N/kg. lactalbumin hydrolysate	5	196 ± 11	100 ± 4
0.35 g. N/kg. lactalbumin hydrolysate + 1% CHA [c]	5	206 ± 8	120 ± 6
0.35 g. N/kg. casein hydrolysate	5	210 ± 12	127 ± 8

[a] TCA = Total circulating albumin in per cent of the amount determined prior to protein feeding with standard error after 4 weeks of feeding.

[b] TCG = Total circulating globulins in per cent of the amount determined prior to protein feeding, with standard error, after 4 weeks of feeding.

[c] CHA = The fraction of casein hydrolysate soluble in 60 per cent alcohol but insoluble in 80 per cent alcohol.

line vitamin B_{12}-like activity using Lactobacillus lactis Dorner likewise showed the presence of only a minute amount of this factor (less than 1.0 mg. per g. of this fraction). Nevertheless, supplementation of soybean diet with this fraction to the extent of one part per hundred thousand stimulated the growth rate (Table X) of weanling rats raised by mothers fed on soybean diet. The addition of crystalline vitamin B_{12} to a dose giving a maximum response, accentuated the rate growth even further. This fact may be considered as an additional evidence that this factor is distinct from vitamin B_{12}.

TABLE IX

Some Chemical and Biological Characteristics of the Casein Hydrolysate Fraction, Soluble in 60 Per Cent Alcohol but Insoluble in 80 Per Cent Alcohol

Analyses	Results	Method	Reference
Total N as % of dry weight	14.6	Micro-Kjeldahl	—
Amino nitrogen as % of total nitrogen	68	Ninhydrin reaction	Dillon (31)
Microbiological assays			
(a) for LLD	traces	LLD	Shorb (32)
(b) for strepogenin	none	L. caseii	Sprince and Woolley (33)

TABLE X

The Effect of the Fraction Obtainable from Casein Hydrolysate on the Growth Rate of Young Rats

Diet	Supplement	Amount added mg./100 g. ration	Increase in body weight over control (in g.) 1 week	2 weeks	3 weeks
Soybean	Casein factor	0.5	5	12	18
Soybean	Casein factor	1.0	8	14	22
Soybean	Casein factor	2.0	8	15	21
Soybean	Casein factor Crystalline B_{12}	2.0 1.0 (micrograms)	14	23	38

Soybean diet: 60 per cent soy bean meal
30 per cent sucrose
4 per cent salt mixture
6 per cent mazola

III. The Effect of Dietary Proteins on Repletion of Liver Proteins

The works of Addis (34), Kosterlitz (35), Long (36), and their associates demonstrate that when animals such as rats were allowed to fast or given a protein-free diet, liver suffered a very outstanding loss in proteins. This loss was likewise accompanied by a decrease in the enzyme content such as cathepsin, dehydrogenase, etc., which could be restored on protein repletion (Miller, 37). Chow and his associates (38) reported that severe protein depletion brought about a decrease in one of the as yet unidentified nutritional accessory factors. The factor stimulated the growth of Streptococcus fecalis Rough in a medium containing adequate amount of crystalline vitamin B_{12}. It also corrected the growth-retardation effects of sulfasuxidine in young rats. This trace nutrient differed from vitamin B_{12} chemically in that its microbiological activity could be destroyed by Wilson's purified trypsin (1:280) which did not affect the activity of vitamin B_{12}. The destructibility of the microbiological activity by proteolytic enzyme suggests the protein nature of this factor.

The microbiological activity in a fresh liver homogenate was markedly increased by some 10 or more fold, if it were allowed to autolyze at 37°C. for approximately 48 hours. It is therefore of interest to ascertain whether different dietary proteins are equally effective in replenishing this liver factor. Results (39) show that when depleted rats were repleted with different proteins, the number of the microbiological units per g. of fresh liver in the saline extract after incubation differed according

to the protein fed (160 ± 4 for casein; 100 ± 6 for wheat gluten; and 60 ± 9 for egg white).

The results indicate, therefore, that the ability of a given dietary protein to stimulate the synthesis of tissue protein or of a nutritional factor like the one mentioned above does not necessarily correlate with the orthodox growth efficiency tests. It would be interesting to ascertain whether dietary proteins, likewise, play a role in the synthesis of liver proteins, similar to the studies on plasma proteins. Such investigation, however, does not appear profitable at this time because of the lack of a suitable means of identifying the protein components. However, excellent starts have already been made by numerous investigations (40, 41, 42, 43) in an attempt to identify, either by means of chemical fractionation or by electrophoretic analyses, the various protein components present in the liver of normal and protein-depleted rats. The results are uncertain because no adequate means has yet been found to stop the enzyme actions present in liver which constantly alters the protein moiety by autolysis during analyses.

IV. The Role of Dietary Proteins in the Hypertrophy of Gonads

The study on the ability of dietary proteins to support growth is usually made by observing the growth effect of the whole body, the assumption being that the organs will grow in proportion to the body weight. Recently Yamamoto and Chow (44) investigated the effect of proteins on the growth of tissues of the gonads of young normal rats in response to the added stimulus of the injected gonadotrophic hormones, not derived from the pituitary. In this study, 23-days old rats of both sexes were fed *ad libitum* 5 diets containing 20 per cent proteins of different nutritive values (casein, lactalbumin, soy bean, wheat gluten, and stock diet). A sixth diet containing no protein was also included. On the 27th day, to one-half of the rats in each group was administered subcutaneously, a daily dose of 10 units of follutein [3] to male rats and of 0.10 unit of gonadogen [4] to female rats. The remaining animals served as dietary controls. All animals were sacrificed on their thirty-third day. Their sex organs were removed, weighed, and analyzed for total nitrogen with the micro-Kjeldahl method. The results of the experiment are summarized in Table XI. They demonstrate that the

[3] Follutein is a commercial preparation (E. R. Squibb & Sons) of sex hormone isolated from the urine of pregnant women.

[4] Gonadogen is a purified hormone preparation (Upjohn & Co.) derived from pregnant mare serum.

TABLE XI

The Role of Dietary Proteins on the Synthesis of Tissues of the Gonads of Rats Injected with Gonadotrophin

Diet		Males					Females				
		Gain in body weight (g.)	Prostrate		Seminal Vesicles		Gain in body weight (g.)	Uterus		Ovaries	
			mg.	mg. % [a]	mg.	mg. % [a]		mg.	mg. % [a]	mg.	mg. % [a]
Lactalbumin	E [b]	16.4	81.8 ± 11.8	134	54.1 ± 9.8	89	17.5	130.8 ± 31.1	225	36.7 ± 9.5	63
	C [c]	25.8	28.0 ± 8.4	40	10.0 ± 2.8	14	22.9	40.9 ± 14.5	64	18.7 ± 4.4	29
Casein	E	23.6	92.0 ± 10.1	138	54.0 ± 9.3	81	22.5	135.3 ± 28.8	218	34.8 ± 6.3	56
	C	34.7	33.5 ± 10.1	41	12.3 ± 2.7	15	28.8	46.8 ± 8.5	67	17.8 ± 2.5	26
Stock	E	41.1	96.3 ± 16.2	115	55.6 ± 15.0	66	34.8	182.7 ± 50.7	238	36.7 ± 7.1	48
	C	44.6	35.7 ± 8.6	40	12.7 ± 2.6	14	36.7	54.7 ± 15.6	69	18.1 ± 3.2	23
Soybean	E	33.5	86.0 ± 12.6	113	53.8 ± 7.2	70	32.3	180.9 ± 54.7	254	38.6 ± 7.3	54
	C	41.9	37.4 ± 10.6	43	11.5 ± 2.6	13	34.7	69.7 ± 14.9	93	18.9 ± 6.3	25
Wheat gluten	E	0.2	60.9 ± 9.3	142	35.4 ± 8.1	83	1.2	133.2 ± 26.2	314	27.0 ± 7.4	64
	C	0.6	13.9 ± 3.2	32	6.7 ± 1.5	15	3.0	23.9 ± 5.3	54	13.9 ± 3.0	31
Protein-free	E	—8.0	43.7 ± 8.9	126	28.1 ± 9.2	81	—7.7	120.4 ± 28.6	365	33.2 ± 12.7	100
	C	—6.9	9.8 ± 2.4	26	5.6 ± 1.3	15	—7.3	18.5 ± 4.8	57	10.9 ± 2.0	33

[a] Organ weights expressed in mg. of tissue per 100 g. body weight.

[b] E = Experimental rats injected with gonadotrophin.

[c] C = Control rats receiving no gonadotrophin.

injection of gonadotrophin produced several fold increase in the sex organs including those on protein-free diet. Such growth obviously took place at the expense of other body tissues. Injection of gonadotrophin produced hypertrophy of gonads but a loss in body weight. The degree of hypertrophy was dependent on the nutritive value of the dietary proteins. The ratio of the weights of the gonads to body weight at necropsy were significantly different among the groups of rats. These data suggest that the response of normal rats to the stimulus of sex hormones is dependent on the protein intake.

Similar studies (45) were made by following the growth of hypophysectomized rats injected with growth hormone and fed on different proteins. Five groups of normal rats, hypophysectomized on their twenty-third day, were fed isocaloric diets containing 30 per cent test proteins, 32 per cent carbohydrates, 24 per cent fat, 2 per cent cod liver oil, 8 per cent yeast, and 4 per cent of a salt mixture. The animals were given a daily injection of 0.1 mg. of a purified growth hormone preparation, isolated from beef pituitary. The data (tabulated in Table XII)

TABLE XII

Food Consumption and Body Weights of Hypophysectomized Rats Injected with Growth Hormone and Fed Different Proteins

Protein fed	Weeks of feeding: 0 F.C.[a]	0 Av. wt. in g.	1 F.C.[a]	1 Av. wt. in g.	2 F.C.[a]	2 Av. wt. in g.	3 F.C.[a]	3 Av. wt. in g.
Protein-free	0	43.7	35	40.0	—	—	—	—
Soya protein	0	45.9	44	55.9	26	57.7	7.6	59.4
Crude casein	0	47.1	56	63.2	30	68.5	34	71.9
SMA vit.-free casein	0	45.5	47	61.3	24	66.3	25	67.5
Lactalbumin	0	44.8	40	58.9	22	64.0	20	68.8

[a] F.C. = average weight in g. of food consumed per rat per week.

demonstrate that animals on a protein-free diet failed to survive for even one week in spite of their consumption of relatively adequate amount of calories. The weights of animals on the test protein diets increased significantly. The increase was the smallest for soybean proteins and the greatest for the crude casein diet. The animals consumed essentially the same amount of food during the first two weeks. The authors concluded that the difference in growth response of hypophysectomized rats may be explained not on the basis of the amino acid content but on the contaminating nutritional factor in the test proteins.

V. Discussion

The classic studies of Rose and others demonstrated beyond doubt that synthesis of tissue proteins requires the presence of all the so-called essential amino acids in adequate quantities. The requirement for these acids varies from one species of animals to the other and is also dependent on the criterion used. Thus, according to Burroughs, Burroughs and Mitchell, lysine may be considered essential for growth of young rats, but it is not needed for the maintenance of nitrogen equilibrium in the normal sexually mature rats. Although the conclusion on the essentiality of lysine [5] has been disputed, there is a general agreement that from the quantitative consideration, an amino acid may be considered essential, if synthesis of this given acid cannot keep pace with the requirements, be it for growth or for maintenance. Since the synthesis of amino acids can involve the use of the so-called nonessential ones, the supply of the latter acids must play a role in ascertaining the indispensability of amino acids.

The synthesis of a complex protein molecule is obviously dependent on the presence of all the essential amino acids. The importance of the time factor has been demonstrated in two types of experiments. Firstly, the intravenous injection of tryptophane to dogs previously fed on acid hydrolysate of casein, deficient only in this essential amino acid, can bring about nitrogen retention, which otherwise will not take place. However, the supply of tryptophane must not be unduly delayed. Secondly, it was shown that if animals were offered simultaneously two deficient proteins, which can adequately supplement one another for growth requirements, such a mixture can be readily utilized for the synthesis of body tissues. If the second protein were given long after the first one had been ingested, however, the animal received no benefit from the mixture. The studies of Harte (12) and Geiger (13) also provide additional evidence that "incomplete" amino acid mixtures or polypeptides are not stored in the body.

The importance of availability of all the essential building stones at appropriate time and at the site of synthesis was also recognized by Melnick (46) in his early reports. He postulated that the nutritive value of an intact protein depends not only on the presence of the essential amino acids but also on the relative rate at which they are released by the enzymes. Thus, according to his hypothesis, the increase of biological value of soy bean protein after heating is due to the greater ease

[5] For a discussion on the discrepancy of results, the readers are referred to: "Studies in Amino Acid Utilization" by Wissler, R. W., Steffee, C. H., Frazier, L. F., Woolridge, R. L., and E. P. Benditt, *J. Nutrition* 36, 245 (1948).

of liberating the limiting amino acid methionine as a result of heat treatment, thus making this acid available for use.

The parts which the above factors play in the protein synthesis cannot be over-emphasized. In our present communication, the evidences for the possible existence of yet another factor which plays a role in the building of tissue proteins, are presented. In the first place, it was shown that the intake of certain dietary proteins can influence the synthesis of the type of tissue proteins. For example, wheat gluten which is inferior to egg white proteins for the promotion of growth of weanling rats, is at least equally as good for the repletion of the liver of rats after 2 to 3 days of inanition. Secondly, when young normal rats are administered a sex hormone so as to stimulate the hypertrophy of the gonads, the dietary protein again plays an important role in the synthesis of these selected tissue proteins. This phenomenon is again demonstrated by the fact that the growth of hypophysectomized rats under the stimulus of growth hormone is dependent on the dietary proteins and probably on the nutritional factor. Lastly, it was found that feeding of casein hydrolysate to protein-depleted dogs stimulates the production of both albumin and globulins whereas feeding of lactalbumin favors the regeneration of albumin. This difference in the plasma regeneration property cannot be adequately accounted for by the difference in the amino acid composition of the two milk proteins, nor in the amounts of protein nitrogen retained by the animals. Fractionation of casein hydrolysate with alcohol at low temperature produced a crude fraction which, when added to lactalbumin hydrolysate in a minute amount, will now stimulate the production of both albumin and globulins like casein or its hydrolysate. This fraction contains no strepogenin and only traces of the x-factor of Hartman (47). It will stimulate the growth of weanling rats produced by mothers on soybean diet, though not as effectively as the crystalline vitamin B_{12}.

The hypothesis that the synthesis of tissue proteins is dependent on the presence of a nutritional factor or factors receives a further support in that dogs so severely depleted of their protein reserves as to cause a sharp decrease in the total circulating alpha globulins, cannot utilize nitrogen from an otherwise adequate casein hydrolysate, and will ultimately end in death. However, injection of liver extract at the appropriate time can restore the ability of the animal to retain the dietary nitrogen derived from the hydrolysate.

The possibility of the existence of a "directive substance" for synthesis is not without precedent. It has been demonstrated (48, 49, 50) that the addition of a substance present in the extract of Type III Pneumococcus can transform the rough strain derived from Type I

Pneumococcus, to Type III smooth organism. Thus, they were able to affect the synthesis of the encapsulating polysaccharide by the addition of the so-called transforming principle (51, 52). Later, McCarty and Avery (53, 54) isolated this substance in a chemically homogeneous form and identified it to be a desoxyribose nucleic acid. The existence of a substance capable of directing the synthesis of tissue proteins from the building stones derived from our dietary proteins can be unequivocably proved only after the isolation in a pure form.

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The Protein and Amino Acid Requirements of Man *

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CONTENTS

I. Species Differences in Protein Needs 116
1. Immature Animals 116
2. Adult Animals 117
II. Qualitative Amino Acid Requirements of Various Species 118
1. Growing Rats 118
2. Young Mice 118
3. Chicks 118
4. Infants 119
5. Adult Rats 120
6. Dogs 121
7. Young Adults 121
III. Quantitative Amino Acid Requirements of Rat and Man 122
1. Immature Rats 122
2. Infants 122
3. Young Adults 124
IV. Growth and Protein and Amino Acid Needs 127
V. Effect of Nutritional State on N-Requirements 128
1. Factors Effecting Rate of N-Depletion 128
2. The Effect of N-Depletion States on Protein and Amino Acid Requirements 130
VI. Metabolic Interactions of Essential and Nonessential Amino Acids . . 132
VII. Influence of Calories on N-Equilibrium and Amino Acid Requirements . 133
1. Effect of Carbohydrates 134
2. Protein Sparing Value of Various Carbohydrates 134
3. Effect of Dietary Fats on Protein Metabolism 135
4. Calories and Protein Metabolism 136
VIII. Influence of Vitamins on N-Metabolism 136
1. Methyl Group Metabolism 136
2. Nicotinic Acid-Tryptophan Metabolism 137
3. Relation of Vitamin C to Tryosine Metabolism 137
4. Pyridoxine and Amino Acid Metabolism 138
5. Other B-Vitamins and Protein Metabolism 139
IX. Relation of Hormones, Proteins and Amino Acids 140
1. Biosynthesis of Hormones 141
a. Phenolic Derivatives 141
b. Proteins 141

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c. Steroids 141
2. Anabolic and Catabolic Reactions 142
X. Summary 146
References 147

I. Species Differences in Protein Needs

1. Immature Animals

Although considerable information has been obtained on the protein and amino acid needs of rats, dogs, and other animals, relatively little is known regarding the human needs for these life sustaining nutrients which constitute the most essential moiety of every body tissue. The species differences in this respect are both of a qualitative and quantitative nature.

As early as 1874, Bunge (1) suggested that the chemical composition of milk in different species is correlated with the chemical composition of the new born animal. In the light of newer measurements, this correlation, if it does exist, does not appear to be striking. But, the parallelism between the protein content of milk, and rate of growth of the new born mammal, indicated in Table I, remains valid and most interesting (2).

TABLE I

Relation of Protein Content of Milk and Growth of Newborn of Various Mammals

Species	Time required for newborn to double body weight, days	Protein content of milk, per cent
Man	180	1.6
Horse	60	2.0
Calf	47	3.5
Kid	19	4.3
Pig	18	5.9
Lamb	10	6.5
Dog	8	7.1
Cat	7	9.5

Despite the quantitative differences in composition of the milk of different species, man and most farm and laboratory animals generally thrive on cow's milk. Some interchanges, however, have not proved completely satisfactory. While kids make the same growth per calorie on cow's as on goat's milk (3), calves are said to react unfavorably to goat's milk, and while foals are said to react unfavorably to undiluted

cow's milk, they thrive on undiluted goat's milk; even though goat's and cow's milk have the same quantitative composition. Fresh cow's milk is not satisfactory for feeding very young puppies, partly at least because the concentration of protein, fat, and minerals in cow's milk is so much lower; and the sugar so much higher than it is in bitch's milk (4). Young kittens have been observed to reject and thrive poorly on fresh milk, but accept and grow well on evaporated milk which contains proteins in double the amount of fresh milk (5). More recently, Scott and Norris (6) have shown that human milk is entirely unsuitable as a food for the rat. In addition to the limiting nutritional factors which may arise from protein concentration and amino acid composition of the proteins, the poor biological value of various milks in diverse species may be associated with differences in permeability of the digestive tract lining to proteins of heterologous milk and consequent allergic reactions (7).

2. Adult Animals

An interesting discussion of some nutritional differences of the various adult mammals can be found in Williams' monograph, "Appraisal of Human Dietaries by Animal Experiments" (8). Some pertinent examples are worth mentioning here.

Dr. Jet C. Winters reported at this conference that the growth of rats maintained on the so-called Average American Diet approached normal, but mated at maturity, the females were often sterile. Their young frequently failed to survive and those that did so were usually stunted at weaning. There were many stillbirths and many young were eaten by the mothers. Lactation appeared poor. A companion experiment with chicks indicated serious defects of such human diets for the growth of that species also.

Dr. H. E. Robinson reported at this conference that considerable mammalian species variation in nutritional requirements for reproduction prevail. After dozens, if not hundreds of experiments, he concluded that the rat is not a satisfactory test animal for canned dog or cat food, nor is the dog a satisfactory test animal for the cat food.

Obviously, the physiological basis for the nutritional differences of mammals are multiple. Prime consideration must be given to variations in the net dietary energy required for growth of the young and subsequent maintenance of the adult structure, variations in the rate of growth of organs and ultimate relative size of these organs, and finally the presence of special structures, *e.g.*, hair, horns, mammary glands.

II. Qualitative Amino Acid Requirements of Various Species

1. Growing Rats

In 1938, Rose (9) stated that "Animals (rats maintained on a diet containing only 10 essential amino acids) gained in weight just as rapidly as when all of the protein components were supplied preformed" also "that the omission of any or all of the dispensable amino acids from the ration exerts no inhibitory action." Supporting experimental data for these statements has not as yet been published. However, from the experimental results which have appeared from other laboratories (10) it definitely appears that amino acid mixtures containing only these 10 essential amino acids in the most optimal proportions will only support subnormal growth of immature rats. If the mixture is not of optimal composition, however, complete or partial growth failure will be experienced. Improved growth has been achieved by the addition of unessential amino acids (11). Furthermore, normal growth in rats and mice comparable to that attained with complete enzymatic digests is observed only with the addition of polypeptide fractions (12). It appears at this writing that in rats maximal growth with synthetic diets can be obtained only with those containing proteins of good quality.

2. Young Mice

By feeding mixtures of the purified compounds, Bauer and Berg (13) have established that methionine, phenylalanine, valine, leucine, isoleucine, and threonine are required for the growth of mice. Previous investigations by other workers have shown the need of this animal for tryptophan, histidine and lysine (14). Bauer and Berg observed no difference in the growth rate on diets with or without arginine and concluded that the mouse is able to synthesize this amino acid. These investigators also noted that the mixture containing only the essential amino acids is inferior nutritionally to a complete amino acid mixture. Subsequently, Woolley (15) demonstrated that when mice were fed a highly purified ration in which casein hydrolysate plus tryptophan and cystine was the nitrogen source, they grew at a submaximal rate. The addition of a polypeptide fraction, strepogenin, effected a stimulation of growth comparable to that obtained with intact protein.

3. Chicks

Recent critical studies by Almquist and Grau (16) have shown that in marked contrast to the rat, the chick cannot synthesize arginine at

all, and must obtain all its need of this amino acid from the diet. Moreover, the chick apparently cannot synthesize glycine, glutamic acid, and porline at a rate commensurate with the demands of early rapid growth. Under certain conditions, the chick appears to benefit from the presence of cystine and tyrosine in the diet.

4. Infants

Studies by the author and his associates have shown that tryptophan (17), isoleucine (18), methionine (19), and lysine (20) are essential for the maintenance of physiological growth and N-retention of infants from 3 months to one and a half years of age. Unpublished qualitative studies wherein determinations of the stimulatory effect on growth and N-retention of supplements of threonine, leucine, phenylalanine and valine on evaporated milk formula, fed at levels which reduced the intake of these amino acids to suboptimal amounts, suggest that these amino acids are also dietary essentials for children of this age group.

Feeding experiments of 10 days duration with a synthetic formula containing a lysine-reinforced enzymatic digest of lactalbumin made poor in basic amino acids by elution through columns of Amberlite IR 100 (sodium cycle operation) indicate that arginine and histidine are required preformed in the diet in very small amounts or not at all by the infant (21). Similar short term studies indicate that a dicarboxylic acid poor enzymatic casein digest (prepared by passage of 5 per cent solutions through Amberlite IR 4 columns) can maintain normal infants in normal N-retention and body weight gains. Although it would seem from these observations and correlative data that the infant requirements for arginine, histidine, and glutamic and aspartic acids for the sustenance of good nutrition are negligible, it remains to be demonstrated whether infants can thrive normally for a long period of time on diets completely lacking in these amino acids. Such a demonstration would be difficult and of little practical value, since these 4 amino acids occur in relatively large amounts in proteins of the usual infant foods. The low glycine content of casein and casein preparations which we have employed as the principal source of N in various test diets on which infants have thrived well, suggests that the exogenous need for this amino acid must be negligible, if at all extant. Direct information on the alanine, proline, hydroxyproline, and serine needs of the infant have yet to be ascertained. The available data on this topic are summarized in Table II.

TABLE II

Classification of Qualitative Amino Acid Needs for Growth and N-retention in Various Species

Chicks (Almquist)	Rats and dogs (Rose)	Infants (Albanese)	Young male adults (Rose)
Required for growth:	*Essential:*	*Required for growth and normal N-retention:*	*Essential for N-equilibrium:*
Arginine	Arginine	Isoleucine	Isoleucine
Glutamic acid	Histidine	Leucine	Leucine
Histidine	Isoleucine	Lysine	Lysine
Isoleucine	Leucine	Methionine	Methionine
Lysine	Lysine	Phenylalanine	Phenylalanine
Methionine	Methionine	Threonine	Threonine
Phenylalanine	Phenylalanine	Tryptophan	Tryptophan
Threonine	Threonine	Valine	Valine
Tryptophan	Tryptophan		
Valine	Valine	*Required with certain proteins:*	*Non-essential for N-equilibrium:*
Required under certain conditions:	*Unessential:*	Cystine	Alanine
Cystine	Alanine	Tyrosine	Arginine
Tyrosine	Aspartic acid		Aspartic acid
	Cystine	*Probably not required:*	Citrulline
Not required for growth:	Glutamic acid	Arginine	Cystine
Alanine	Glycine	Aspartic acid	Glutamic acid
Aspartic acid	Hydroxyproline	Glutamic acid	Glycine
Hydroxyproline	Proline	Glycine	Histidine
Proline	Serine	Histidine	Hydroxyproline
Serine	Tyrosine		Proline
		Dispensability not tested:	Serine
		Alanine	Tyrosine
		Hydroxyproline	
		Proline	
		Serine	

5. *Adult Rats*

In adult animals where rate of growth can no longer be employed as a criterion, the N-balance technique is a valuable tool, albeit equivocal, in ascertaining the nutritive role of individual amino acids. Wolf and Corely (22), using 9 of the amino acids essential for growth of immature rats, omitting arginine, found that the adult animal could be maintained in N-equilibrium; but that omission of any one of these, induced N losses. They conclude therefore, that with the exception of arginine, the qualitative amino acid requirements of the adult rat were the same as those of

the young. Burroughs, Burroughs, and Mitchell (23) found that only threonine, isoleucine, valine, tryptophan, and methionine are needed to restore the endogenous nitrogen loss in the adult rat. They also found tyrosine and phenylalanine to be interchangeable and leucine, histidine, and arginine to be dispensable; the omission of leucine must be corrected by the addition of norleucine. Lysine appeared to be dispensable by these tests. This seemingly anomalous result may have been due to the fact, not then known, that because lysine is the only amino acid which does not participate in exchange reactions (24), the lysine reserves are consequently depleted very slowly and by the same token, the maintenance requirements are relatively low.

Wissler and his associates (25) have recently measured the qualitative amino acid requirements for maintenance of the adult male rat in terms of preservation of appetite and body weight, as well as N-balance, and found that the 9 essential amino acids required for growth of the immature are also necessary for maintenance of the adult animal.

6. Dogs

It is of historical interest to observe that the dietary indispensability of tryptophan was first ascertained in dogs by Abderhalden about 40 years ago by feeding acid hydrolyzed casein as the principal N component of the diet (26). More complete measurements of the qualitative amino acid requirements of adult dogs have been reported abstractedly by Rose and Rice (27). It is claimed by these workers, that their animals could be maintained in positive N-balance on the same 9 amino acids essential for rat growth. More recent studies by Madden and others on this subject will be discussed in another section of this monograph.

7. Young Adults

The qualitative need for dietary tryptophan and lysine for the maintenance of N-balance in young adults was demonstrated by experiments performed by the author and his associates at Johns Hopkins University in the summer of 1941 (28, 29). A simultaneous experiment showed that arginine was not required for maintenance of N-balance (30). The following summer, our group performed further experiments which confirmed the earlier observations and demonstrated further that methionine must be present in the diet in order to maintain N-equilibrium (31). The role of cystine could not be demonstrated conclusively by these experiments, but was proven dispensable by later experiments (32). Subsequent studies performed by us (33) in the summer of 1943, showed

that dietary histidine, like arginine, is not needed for the maintenance of N-balance of adults.

Subsequent and concurrent studies by Rose and associates (34) reported as abstract notes, indicated that threonine, valine, leucine, isoleucine, and phenylalanine are qualitatively necessary for the re-establishment of N-equilibrium in young male adults.

III. Quantitative Amino Acid Requirements of the Rat and Man

1. Immature Rats

After the qualitative determinations of the essential amino acids, Rose and coworkers (35) set out to ascertain the quantitative requirements of the immature rat. These amounts were established for rats by feeding them diets in which the percentage of one amino acid was increased until a further increment gave no additional rise in the growth rate. The values given in Table III have been reported without supporting data.

TABLE III

Minimal Amount of Each Essential Amino Acid Necessary for Normal Growth of Immature Rats When the Nonessentials are Supplied

Amino acid	*Per cent*
Lysine	1.0
Tryptophan	0.2
Histidine	0.4
Phenylalanine	0.7
Leucine	0.8
Isoleucine	0.5
Threonine	0.5
Methionine	0.6
Valine	0.7
Arginine	0.2
	5.6

The quantitative amino acid requirements of the adult rat and other experimental animals, immature or mature, do not appear to have been investigated as yet.

2. Infants

A survey of the literature indicates that on a body weight basis, the nitrogen needs of the infant as provided by the usual cow's milk formula, are 4 to 5 times higher than those of the adult (36). The available data, however, do not reveal whether these higher protein requirements of the

infant arise from a proportionate increased need for all the amino acids or from a limiting effect created by greater demands of the growing organism for one or several of the amino acids. The lack of, and the need for this information which is of obvious importance to practical infant dietetics and the physiology of growth in general, (*e.g.*, benign and malignant neoplasms) prompted the author and his collaborators to undertake a continuing program for the study of the amino acid needs of the infant and the growing child. In all of these studies, the requirement of an essential amino acid was estimated from the additional amount needed to restore to physiological levels the N-retention and rate of body weight gain of subjects previously maintained for short periods on diets poor in the test amino acid. The diets contained approximately 100 calories per 100 g., the percentage caloric distribution being as follows: protein, 14; fat, 36; carbohydrate, 50. The protein moiety consisted in all instances of proteins or protein preparations lacking one of the essential amino acids. The observations were all made on normal male children who were given the diets in 5 feedings daily at the rate of approximately 100 calories and 0.56 g. N per kilo of body weight and 50 mg. of ascorbic acid together with 15 drops of oleum percomorpheum daily. The diet periods were of 7 days duration and consecutive. The infants were weighed daily during the course of the experiment. The data on N-retention were calculated from the results of N determinations of the 24-hour urine collections, analysis of the pooled feces for each period, and from computations of the daily nitrogen intake based on food consumption records and the known N content of the diets. Measurements of the Hb, total plasma proteins, albumin, globulin, and NPN were done during each period interval.

On the basis of changes in rate of weight gain and nitrogen retention induced by fractional supplementation of the tryptophan-deficient diet, it was estimated that the infant requires approximately 30 mg. of l (−) tryptophan per kilo of body weight per day (17). This value is approximately 5 times the adult tryptophan requirement previously reported by the author (37), and suggests that the high milk protein need of the infant is predicated in part by the tryptophan requirement.

In experiments of similar design using acid hydrolyzed beef hemoglobin as the isoleucine poor moiety of the diet, it was found that the normal infant requires approximately 90 mg. of L-isoleucine per kilogram per day, which is approximately one-third the quantity provided by the usual evaporated milk formulae (18).

The cystine and methionine requirements of infants were determined by using a diet deficient in these two amino acids as the principal source of nitrogen which was supplemented with varying proportions of L-cys-

tine and L-methionine. Thus it was found that infants who received no cystine supplements required 85 mg. of L-methionine for the attainment of normal N-retention and body weight gains. The infants who received diets containing 1 per cent L-cystine, required only 65 mg. of L-methionine for restoration of the growth criteria to physiological levels (19).

On the basis of results obtained with a fractionally L-lysine supplemented wheat gluten diet, it appears that the infant requires some 140 to 200 mg. of L-lysine per kilo per day for the establishment of normal growth vectors (20).

In recent preliminary observations with the "saturation point test" wherein the amino acid requirements are measured in subjects who are maintained in normal N-metabolism by natural foods fed *ad libitum,* rather than in depleted subjects, we have found that 3 months old children need 205 mg. per kilo of L-phenylalanine per day. Six to eight months old children require 170 mg. per kilo of L-phenylalanine per day. In older children, ranging in age from 2 to 8 years, it was found that the phenylalanine needs per kilo of body weight decreased progressively with age from 170 to 100 mg. (38).

From data available on the biological value of various proteins and protein products in the infant, and analytical data on their composition, it is possible to make valid calculations of the minimal quantities of each amino acid needed for good nutrition when all of the amino acids are provided in the diet. The results of these calculations are collected in Table IV (39). The minimal quantities given in the last column should be considered as useful approximations, pending more exact determinations.

3. Young Adults

In the course of making a qualitative assessment of the essential amino acid requirements of medical students, we were able to make some quantitative measurements of these nutriles. By the stepwise addition of L-tryptophan to a tryptophan-deficient diet, containing all the other amino acids, it was found that 6.0 to 9.0 mg. per kilo were required to restore N-equilibrium and only 3.0 to 6.0 mg./kilo/day to reestablish normal tryptophan excretion in subjects previously depleted (37).

Experiments with a diet deficient in both S-amino acids showed that a supplement of 19 mg. of DL-methionine was needed to reestablish N-equilibrium in adult males. Since these diets contained 10 per cent of extraneous N which can be reasonably assumed to contain 1.0 and 3.0 per cent of tryptophan and methionine, respectively, it would seem that a total of 7.0 mg. of tryptophan and 21 mg. of methionine are needed

TABLE IV

Infant Amino Acid Requirements for Normal N-Retention and Body Weight Gains [a]

Milligrams/kilo/day

	Cow's milk products			Beef products				
Amino acids	Evaporated milk	Casein (Amigen)	Lactalbumin (Edamin)	Beef globin	Beef muscle protein	Beef plasma protein	Wheat gluten	Minimal quantity
Arginine	127	160	140	141	206	245	126	126
Histidine	63 [c]	98	81	277	134	112	70	63
Lysine	200	264	304	304	320	342	80 (170) [b]	170
Tryptophan	47	70	87	59	93	49	30	30
Phenylalanine	177	199	213	250	169	182	246	169
Methionine	99	130	98	95	117	86	201	86
Threonine	151	157	206	257	234	245	87	87
Leucine	490	450	556	610	425	356	889	425
Isoleucine	167	251	227	36 (90)	102	126	195	90
Valine	171	263	161	298	227	266	182	161
Tyrosine	172	251	203	160	126	49	234	126
Cystine	27	18	109	18	38	19	46	18
Serine	160	227	172	182	201	—	—	160
Glutamic acid	680	800	470	200	650	588	860	200
Aspartic acid	166	222	340	283	380	370	350	166
Glycine	11	17	0	—	210	—	315	0
Alanine	75	196	98	280	140	—	175	75
Proline	250	286	140	185	210	—	350	140
Hydroxyproline	—	70	—	—	—	—	—	70

[a] These values were obtained from biological value measurements of the author and his associates, and the best amino acid analytical data available for various proteins or protein products employed.

[b] Values in parentheses indicate concentrations of amino acid after supplementation.

[c] Underscored values represent minimal amino acid levels in products tested.

for the maintenance of N-balance in young men when all other amino acids and dietary factors occurring in natural foods are provided (32).

Rose and his associates have also studied the amino acid requirements of male students (40). Their subjects were fed a diet consisting of a mixture of pure amino acids flavored with lemon juice and sugar which was supplemented with wafers made of cornstarch, sucrose, centrifuged butterfat, corn oil, and vitamins. In making quantitative determinations, the requirements of each of the 8 necessary amino acids which constituted the sole source of N were studied by varying the amount of one of the acids in the presence of sufficient quantities of the others. The smallest amount which had to be fed daily to maintain the subjects in continuous positive N-balance was considered to be the minimum requirement. "Furthermore," Rose states, "when several subjects show different requirements for a given amino acid, the highest level must be taken as the minimum." Prior to each experiment, the caloric needs of the subjects were measured so that sufficient energy would be provided to support N-equilibrium. In addition to the 8 essential amino acids, glycine and urea were fed when necessary to keep the N-intake at the necessary levels.

In this manner, the minimum levels for an average 70 kilo adult male were found to be (given in g./day) : L-tryptophan 0.25, L-phenylalanine 1.10, L-leucine 1.10, L-threonine 0.50, L-valine 0.80, L-methionine 1.10, L-lysine 0.80, L-isoleucine 0.70. In the formulation of diets of low amino acid content, Dr. Rose has empirically recommended, however, that double the quantities of these minima be employed. This position is difficult to defend. It should be pointed out here that these recommended allowances for a 70 kg. man are easily met by 20 g. of egg protein or roughly 0.3 g. of this protein per kilo per day. This is to be contrasted with the observations of Murlin and coworkers (41) who found that 1 g. of egg protein/kg./day was the minimal daily requirement.

The values obtained by the Illinois group should also be compared with the human amino acid requirements calculated by Harte and Travers (42) from the data of Bricker, Mitchell, and Kinsman (43) which follow (given in g./day) : arginine, 1.2; cystine, 0.3; histidine, 0.5; isoleucine, 1.2; leucine, 1.7; lysine, 0.8; methionine, 0.5; phenylalanine, 1.4; threonine, 1.0; tryptophan, 0.4; tyrosine, 1.0; valine, 1.1.

In summary, it is clear from the diversity of the data presented in this section that much more work must be done for the establishment of satisfactory quantitative amino acid requirements of man.

IV. Growth and Protein and Amino Acid Needs

The use of isotopes in the study of metabolic processes has shown that the constituents of living cells are involved in continuous biochemical reactions which are primarily concerned with catabolism and anabolism of nitrogenous products (44). Since the process of growth, benign or malignant, can only exist with the predominance of anabolic reactions, it is reasonable to expect that the nutritional support for these reactions must be quantitatively greater than the adult phase in which the anabolism and catabolism have reached equilibrium. Furthermore, since growth usually involves the formation of new structures, and the hypertrophy and atrophy of others which contain proteins having dissimilar amino acid composition, *e.g.*, muscle protein, 7.0 per cent arginine; sperm protamine, 88 per cent arginine, it might also be expected that the amino acid requirements would be qualitatively different from those of the healthy adult. Evidence for this point can be adduced from the data shown in Table V.

TABLE V

Comparison of Dietary Indispensable Amino Acid Needs of the Human Adult and Infant

	Male adults (Rose)		Male infants (Albanese)	
Amino acid	Requirement mg./kilo	Maintenance pattern [a]	Requirements mg./kilo	Growth pattern [a]
Tryptophan	7.2	1.0	30	1.0
Phenylalanine	31	4.3	169	5.6
Lysine	23	3.2	170	5.6
Threonine	14	1.9	87	2.9
Valine	23	3.2	161	5.4
Methionine	31	4.3	85	2.8
Leucine	31	4.3	425	14.0
Isoleucine	20	2.8	90	3.0

[a] Growth and maintenance pattern figures obtained by assigning value of unity to tryptophan.

These amino acid values were calculated from the figures published by Rose (40) for the young adult and those found by us for the infant by direct and indirect measurements. (Table IV). The pattern of these amino acid requirements was obtained by taking the tryptophan figure as unity. It is interesting to note that the lysine ratio in the infant patterns is almost twice that of the adult. This is in accord with Mitchell's recent observations that animals, preponderantly in the anabolic phase, require more lysine than those in N-equilibrium (45). Further examina-

tion of this table discloses that the methionine ratio for the adult pattern is significantly higher than that of the infant. This difference in S-amino acid requirement may be related in part to the relatively greater production of hair which occurs in adulthood (46).

It might be deduced from these considerations that regenerative processes which may be induced in the adult as the result of shock, resulting from surgical procedures or acute illness, may cause a partial quantitative and qualitative shift in amino acid needs of the adult to those characteristic of the growth phase. Similarly, neoplastic processes can be expected to cause a shift in the amino acid needs of the adult (47).

V. Effect of Nutritional State on N-Requirements

1. Factors Effecting Rate of N-Depletion

It has long been known that in starvation the amount of protein metabolized depends principally upon 3 factors, the glycogen content of the individual, the amount of adipose tissue in the body, and the quantity and quality of protein ingested prior to the depletion period. This latter

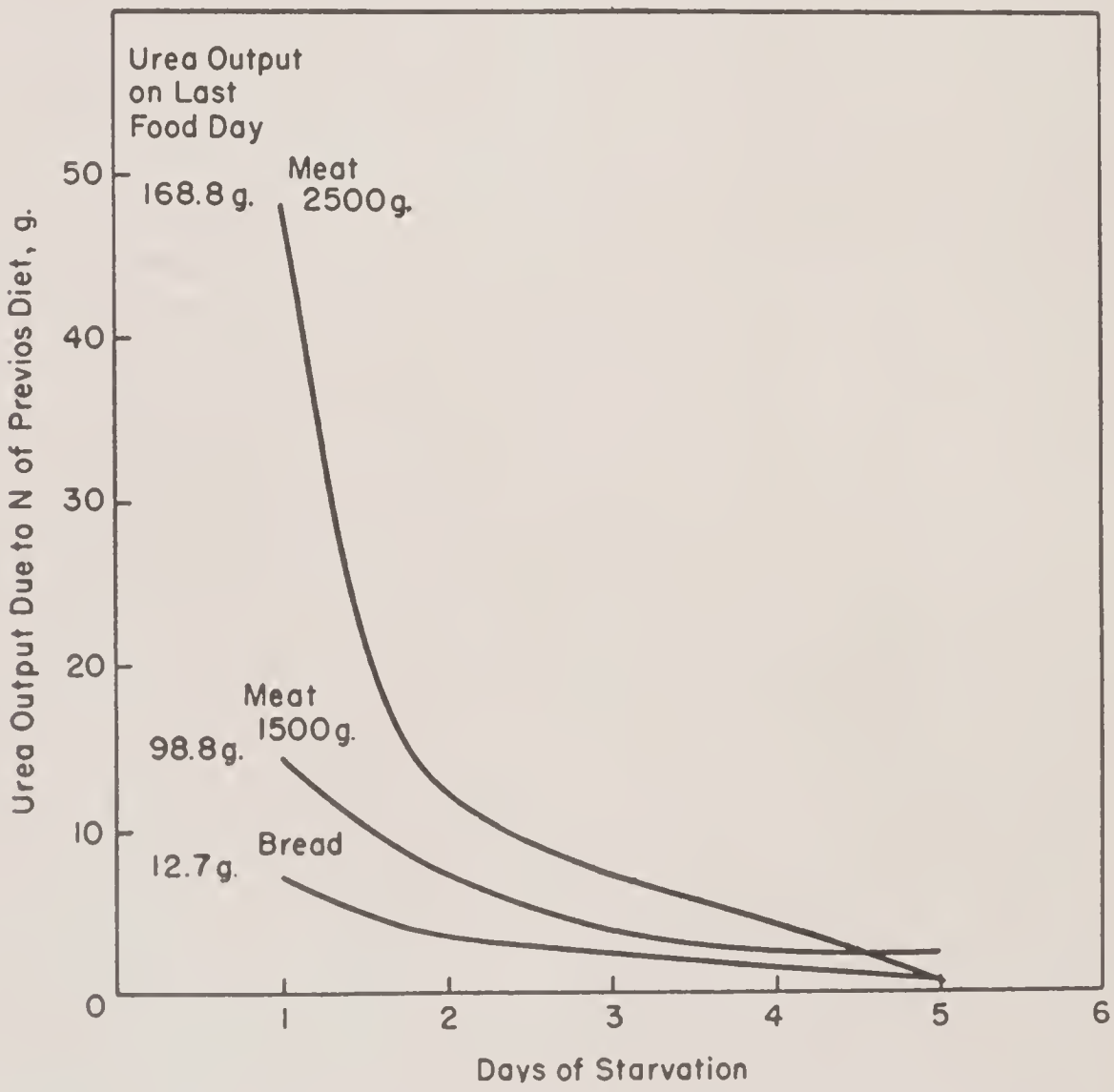

Fig. 1. Effect of previous diet on rate of nitrogen depletion of dogs (data of Voit, 48).

relationship was demonstrated by Voit (48) who fed a dog weighing 35 kg. with different quantities of meat and measured the effect on urea output during the subsequent starvation. By deducting the urea eliminated by the dog on the 6th day when the output became constant at 12.0 g./day from that excreted on previous days of starvation, he obtained the grams of urea derived from the previous food. These data are represented graphically in Fig. 1.

Later experiments with hogs by McCollum (49) showed that the minimum N-expenditure on a protein free starch diet was considerably lowered by feeding zein instead of urea in the foreperiod.

A number of measurements of the minimum endogenous nitrogen metabolism of man have been made and most of the older work has been cited by Millard Smith (50). Table VI has been taken mainly from his paper and it includes the recent data obtained by Bassett (51).

TABLE VI

Minimum Excretion of Nitrogen in Urine of Adult Humans

Investigator	Total urine N, g.	N per kilo, g.	Day of experiment
Folin	2.60	0.0406	12
Klemperer	2.51	0.0395	8
Thomas	2.98	0.0391	19
Graham and Poulton	2.25	0.0366	9
Robinson	1.99	0.0344	11
Klerker	2.01	0.0319	6
Siven	1.84	0.0317	7
Smith	1.58	0.0242	24
Bassett	2.03	0.0372	41 to 45
Bassett	1.39	0.0278	51 to 55 (Testosterone propionate)

Attention is called to the variations in the N per kilo values. Bassett's experiments on the effect of testosterone on the N-minimum suggests that the hormonal state of the individual may exert a more profound influence on the minimum N-metabolism than has hitherto been realized.

Apart from the nutritional history and hereditary factors of the organism, it has been found that although the endogenous N per unit of body weight tends definitely to decrease with increasing weight and age, the endogenous N is constant when related to basal metabolism (52). This correlation seems to prevail not only for man, but for pigs, cattle, sheep, dogs, and rats, and may be due in part to hormonal factors.

2. The Effect of N-Depletion States on Protein and Amino Acid Requirements

Just as the rate of N-depletion is influenced markedly by the state of the protein reserves, so might it be expected that the N-requirements of the organism would be effected by the state of these stores. Available data indicate that less dietary N is needed to maintain the depleted than in the normal state, since the growth potential increases with depletion. Indeed, the degree of depletion has been estimated by the magnitude of positive N-balance that can be produced by a fixed amount of protein (53).

The sparse available data on the amino acid requirements of man, young adult and infant, have been secured for the most part by the depletion procedure. This envolves maintenance of subjects on synthetic diets deficient in the test amino acid until a negative N-balance is induced. The requirement of an essential amino acid is then estimated from the additional amount needed to restore the N-retention and body weight of the subject to physiological levels. Aside from the fact that requirements so measured are valid only for the particular diet complex employed in the study, the values so obtained represent abnormal repletion and not normal maintenance requirements. The difference between the repletion and maintenance values increases with the duration of the period of depletion which we know envolves numerous biochemical readjustments not only of the N, but also of water, carbohydrate, and fat metabolism.

These considerations bring into focus the limitations of the N-equilibrium measurements alone, as an index of good nutrition and the requirements for good nutrition. As long ago as 1900, Siven (54) showed that N-equilibrium could be maintained at a lower level than that ordinarily occurring in starvation. In this experiment which was divided into 5 periods of about a week each, a healthy man weighing 60 kg. who normally ate a mixed diet containing 16 g. of N was given a diet containing 12.7, 10.4, 8.7, 6.3, and 4.5 g. of N, rich in carbohydrate yielding 2444 calories per day, in each successive period. N-equilibrium was established at all 5 levels of nutrition, albeit 3 and 4 days, respectively, were required to achieve it at the lowest N-intake levels. In a subsequent experiment on himself, Siven who was 31 years old and weighed 65 kg. reported that he attained N-balance on an intake of 4 g. of protein N and 2717 calories (55).

Petren (56) maintained a diabetic patient in N-balance for a month or more on a diet containing 4 g. of protein N. Similarly, Chittenden

(57) has found N-equilibrium can be maintained on diets containing but 7.19 g. N and only 21.3 calories per kg.

The author's experience in this connection is illustrated by data shown in Table VII obtained in 1943 on 10 young men from 21 to 23 years of age (58).

TABLE VII

Effect of N-Intake on N-Balance of Young Adult Men

Subject and body weight, kg.	Total urinary N on unrestricted diet, g./day	Restricted Diet (0.1 gm.N/kg.) Total urinary nitrogen, g./day	N-balance g./day	Time required for N-balance, 3-day periods period
BJ, 104	19.4	10.1	+ 0.33	2
VW, 96	17.4	10.0	— 0.55	2
BE, 75	15.8	7.7	+ 0.26	1
YT, 83	10.8	8.3	+ 0.44	2
SL, 94	14.3	10.1	+ 0.86	1
MV, 83	15.5	8.3	+ 1.14	1
WR, 72	6.8	7.2	+ 0.60	1
MR, 80	10.7	7.9	+ 0.65	3
SC, 69	13.7	6.9	+ 0.15	1

It is obvious that amino acid needs measured at lower levels of N-intake are of limited value insofar as the dietary requirements for optimal health are concerned. This is particularly true, if we consider the views held by some that low intake of protein in countries like China and India (in contrast to Australia and the United States where protein consumption is high) is responsible for existing differences in stature, longevity and general health (59). Life expectancy, for example, in Australia and the United States is about twice that of the people of China and India. (Table VIII).

TABLE VIII

Effect of National Diets on Stature and Longevity

	Australia	United States	China	India
Protein N-intake, g./day	18.1	15.7	11.1	9.8
Animal protein, per cent	69	57	8.5	16
Average height, cm.	172	170	158	161
Average weight, kg.	77.2	70.0	54.3	50
Life expectancy, yrs.	65	64	30	27

Doubtlessly, many factors such as sanitation, medical care, heredity, and environment have an effect upon health stature and well being, but there can be no doubt that diet and particularly the protein content of the

diet is a vector in this complex parameter. This view is amply supported by the studies of Cannon and his associates (60) on the relationship of protein metabolism to antibody production and resistance to infection.

VI. Metabolic Interactions of Essential and Nonessential Amino Acids

The early studies of Meyer and Rose (9) in which the growth of rats on a diet containing 11.2 per cent of a mixture of the 10 essential amino acids is stated to have been the same as when 20 amino acids were included in the diet, prompted many to propose and attempt the parenteral and oral use of mixtures of the essential amino acids in the treatment of human malnutrition resulting from surgical treatment or acute illnesses (61). These applications are based on the interesting concept that, because of its "essential nature," such a mixture might have a greater biological value per unit weight than protein products containing all the amino acids. On *a priori* thermodynamic and chemical consideration, it would seem unlikely that a mixture of 10 essential amino acids should approach the nutritional value of a product containing all 20 amino acids. It is important to remember that the administration of a mixture of only the 10 essential amino acids as the sole source of N, imposes two forms of energy losses on the organism (a) energy of decomposition of essential amino acids (EA) to provide radicals for necessary unessential amino acids (NEA) and (b) energy of formation of the unessential amino acids required for protein synthesis.

Moreover, suboptimal protein synthesis might be expected from consideration of the equation: EA / NEA $\rightleftarrows$ Tissue proteins. It is obvious that a temporary deficiency of NEA created by the presence of overwhelming amounts of EA can be expected to decrease the reaction rate to the right. Also, this temporary lack of NEA would seem to favor greater urinary losses of EA and thereby decrease the N-efficiency of the EA mixture.

How are these speculations supported by experiment? Evidence derived from a number of laboratories has shown conclusively that no mixture of the 10 essential amino acids as the sole source of dietary N, can support growth in rats or mice as well as a diet containing protein or protein products (10, 11). Recently, Rose, Oesterling, and Womack (62) have reported on a reinvestigation into the growth of rats on a diet containing only Rose's 10 essential amino acids, 5 of which were supplied in the racemic form. As a basis of comparison, they employed a mixture in which 19 amino acids were included. Thus it was found

that on the diet containing only 10 amino acids, the rats gained only 70 to 75 per cent of the weight, obtained by a diet of all 19 amino acids. Furthermore, it was shown that glutamic acid supplementation of the diet, containing 10 amino acids, greatly improved the growth index of the diet. This greater biological efficiency of the mixture of 19 amino acids is attributed in part to the sparing action of cystine on methionine, of tyrosine on phenylalanine, and proline on arginine requirements of the growing rat.

As mentioned heretofore, we have observed and measured the sparing action of cystine on the methionine needs of the human infant, as well as the phenylalanine sparing action of tyrosine in terms of weight change and N-retention effects. Data are also on hand which suggest that the lysine requirements of the infant may be spared by dietary arginine (20). Evidence recently presented by Rose in studies on the amino acid requirements of young adults, indicates that this sparing action of unessential amino acid which doubtless must occur, cannot be perceived by N-balance measurements. This finding points up the relatively low degree of sensitivity of the N-balance technique as a measure of important metabolic reactions. Although these subtle differences may not be important for the healthy young adult, it should be realized that in nutritional shock resulting from surgical procedure or acute illness when the adult tissues are in a regenerative phase and therefore simulate the metabolic sensitivity of growing tissues of infants, the unessential amino acids may assume a physiological essentiality far above that which can be demonstrated by short term studies on the healthy adult individuals.

In addition to the metabolic interplay of essential and nonessential amino acids; the evidence that whole proteins may contain or carry as an impurity, some nutrient which effects greater utilization of the N-moiety of the diet, must be considered carefully. Whether this effect is due to peptides (strepogenin) as suggested by Woolley (14), or vitamin-like substances of the B-12 variety, or an unknown amino acid, or to a difference in the rate of absorption, need not concern us so much as the obvious fact that any requirement measurements of nutrients made with unnatural diets are of not completely valid measurements for these substances as components of natural foods.

VII. Influence of Calories on N-Equilibrium and Amino Acid Requirements

It has long been known that N-balance can be achieved without carbon equilibrium (2). The carbon and nitrogen contents of the urine

give significant information on the physiological fuel value of protein, carbohydrate, and fat. Normally, every gram of urinary N is accompanied by the elimination of 0.6 to 0.9 g. of carbon. Shifts in the C/N ratio have been found useful in the diagnosis of abnormal metabolic states (63).

1. Effect of Carbohydrates

In 1856 Hoppe (64) reported that carbohydrate ingestion lowered the N-excretion of dogs. This finding was later corroborated by Lusk (2) by a reverse experiment on himself which demonstrated that the withdrawal of 350 g. of carbohydrate from the diet at two levels of N-intake (20.55 and 9.23 g. per day) increased his urinary N-output by 4 g. above the usual values in both instances. This protein sparing action of the carbohydrates was also found to occur in the fasting dog (65) and man (66). These observations bear evidence that the carbohydrates can spare the N-needs of the mammalian organism at all levels of protein intake. The practical implications of this phenomenon were demonstrated by the experiments of Keller (67), Orgler (68), and Rosenstern (69) which demonstrated that the N-retention of infants from a given amount of protein could be greatly augmented by increasing the carbohydrate content of the diet.

2. Protein Sparing Value of Various Carbohydrates

Teleological reason leads to the conclusion that because lactose is the naturally occurring sugar of the milk of all mammals, this sugar possesses nutritive characteristics for nurslings which are lacking in other carbohydrates. These qualities may be associated with the need for galactose in the formation of nerve tissues or the rate of absorption of galactose which is more rapid than glucose or the influence of galactose upon calcium metabolism. Speculations on unique effects of lactose on the N-metabolism have also been entertained. Measurements, by the author and associates, of N-retention of normal infants fed cow's milk formulae supplemented isocalorically with lactose and dextrimaltose do not support these speculations (Table IX).

On the other hand, no untoward effects such as diarrhea or poor assimilation were noted. The fear that lactose supplementation of infant diets may induce cataract formation are unfounded in fact since (a) the incidence of galactosemia is no greater in breast-fed children than in those artificially fed in which other sugars are commonly used, and (b) the cataractogenic effects of lactose in rats are noted only with diets containing 30 per cent or more of the carbohydrate (70, 71).

The nutritional advantages of sucrose and dextrose have not been adequately studied. It may be expected, because of the lability of the fructose molecule, that sucrose would bear biochemical qualities not available with diets containing dextrose alone. Experiments reported by Albanese and associates (72) on the effect of carbohydrate feeding

TABLE IX

Effect of Dextrimaltose and Lactose on N-Retention, and Fecal N of Normal Male Infants

(All results given as averages of 5-day periods)

Subjects	Diets	DMM [a]	DMM	DMM	LM	LM
DS, 3 mos.	N-retention, g./day.	0.89	0.79	0.87	0.68	0.89
4.0 kg.	Fecal N, g./day.	0.50	0.39	0.35	0.61	0.34
RJ, 4 mos.	N-retention, g./day.	0.82	0.52	0.69	0.75	0.38
3.5 kg.	Fecal N, g./day.	0.44	0.40	0.49	0.44	0.40
WM, 8 mos.	N-retention, g./day.	1.32	1.00	0.84	1.14	0.71
7.3 kg.	Fecal N, g./day.	0.60	0.56	0.64	0.49	0.37
FS, 2 mos.	N-retention, g./day.	1.13	1.09	0.95	0.86	0.95
3.3 kg.	Fecal N, g./day.	0.46	0.32	0.34	0.32	0.29

[a] Diet composition: Evaporated milk, 1100 cc.; dextrimaltose #2, 154 g. in DMM; or lactose U.S.P., 150 g. in LM; water to 2640 cc. Both formulae were given at the rate of 3.5 g. of protein and 110 cal. per kilo per day.

on the output of urinary amino acids disclosed that single feedings of 25 g. of sucrose had a greater sparing effect on the N and tryptophan output of young male and female adults than did 25 g. of glucose. More experiments are needed to substantiate the thoughts borne by these too few findings.

3. The Effect of Dietary Fats on Protein Metabolism

The protein-sparing action of fats is considerably less than that of carbohydrates (2). Increasing the fat content of isocaloric diets has been found to result in an increase in rate of weight gain, decrease in heat production, and improved reproductive characteristics of rats (73).

In 1903, Rubner demonstrated that the N-balance of a man fed 23 g. of N and 220 ± 20 g. of carbohydrates could be progressively improved from —3.64 to + 5.75 g. of N by increasing the fat content of the diet from 99 to 350 g. However, it has recently been reported by Consolazio and Forbes (74) that a pemmican diet containing fat, 71 per cent; protein, 27 per cent; and carbohydrate, 2 per cent induced within 9 days in eight men an average weight loss of 5.9 kg. (some of it water), chloride

depletion, marked ketonuria, alteration of glucose tolerance curve, and increased retention of bromosulfalein. No changes in serum protein and nonprotein N, ascorbic acid, cholesterol or basal metabolic rate were noted.

4. Calories and Protein Metabolism

The protein-sparing effect of other nutrients is not only a function of an increased availability of radical, but also dependent upon the energy which these other foods provide. Allison (53) has shown that the N-balance indices of dietary proteins are altered when the caloric intake is reduced to less than 50 per cent of normal. A similar reduction in retention of dietary N with decreased calories has been found in the rat by Willman *et al.* (75) and in man by Schwimmer and associates (76). The latter investigators demonstrated that young male adults fed a quasi-synthetic diet containing egg white retained the dietary N if the intake was 1500 to 1800 or more calories, but did not do so on intakes of 900 calories. Retention of N was obtained, however, at the lower caloric intake if N and fat contents of the diet were sufficiently high. Rose (40) has found that with the use of diets containing mixtures of eight essential amino acids, the caloric intakes had to be increased from a normal value of 40 calories to 55 calories per kg. to maintain N-equilibrium on intake of 10 g. N per day. Others, using isonitrogenous diets containing all 19 amino acids in the form of proteins or protein hydrolysates, have attained N-equilibrium with diets providing 40 to 48 calories per kilo (77). The question might be raised if the increased caloric requirements are not associated with the lack of preformed unessential amino acids, or with the higher S.D.A. characteristics of diets containing only essential amino acids (78).

VIII. Influence of Vitamins on N-Metabolism

Uncertainties regarding the complete human requirements of B-complex vitamins introduces still another hazard in the use of highly synthetic diets for the determination of N requirements. This is so because of the metabolic relationships existing between the amino acids and B-vitamins which will now be discussed.

1. Methyl Group Metabolism

The investigations of du Vigneaud and his associates (79) concerning the methyl group have uncovered many important metabolic relationships between methionine and sulfur metabolism with choline and fat

metabolism on the one hand, and between methionine and choline metabolism with creatine metabolism on the other. That the various methyl compounds of the body such as adrenalin, ergothioniene, anserine, etc., derive their methyl groups from the dietary methionine, choline and betaine is more than a possibility. It is also a well-known fact that certain detoxication reactions involve methylations, *e.g.*, pyridine and nicotinic acid. Cystine, because of its sparing action on the methionine, may well be considered a component of this biochemical system. Recent studies have shown that dietary sources of methyl groups should include both choline and methionine inasmuch as these are not completely interchangeable as sources of methyl in all species. Dependence on methionine alone as a source of methyl for the rat has been found impractical because of the high protein intake (30 per cent of diet) to achieve an adequate methyl supply.

2. Nicotinic Acid-Tryptophan Metabolism

The metabolic relationship of B-vitamins and amino acids was first indicated by the observations of Goldberger and Tanner (80) which demonstrated that systine and tryptophan, as well as large amounts of meat and milk were beneficial in treating pellagra, later shown to be a nicotinic acid deficiency disease. Oral administration of tryptophan or a tryptophan-histidine mixture was also found by Pasha, S. A. (81) to cause rapid regression of pellagra symptoms in man. A clarification of this relationship was achieved by the rat experiments of Krehl and Elvehjem (82) who demonstrated that the pellagragenic action of corn could be remedied by supplementation of the diet with either niacin or tryptophan. They also found more niacin is required by the rat as the tryptophan content of the diet is decreased. The probable conversion of tryptophan to nicotinic acid in man has been demonstrated in adults (83), and in infants (84). More direct proof that tryptophan is a niacin precursor has been achieved by the use of tagged intermediary metabolites (85). The practical aspects of these investigations are found in the elucidation of the long inexplicable fact that a milk diet which is rich in tryptophan can prevent or cure pellagra in infants, even though the amount of niacin provided by milk is little, if any greater, than the amount present in pellagra-producing diets.

3. Relation of Vitamin C to Tyrosine Metabolism

Sealock and Silberstein (86) observed that feeding 0.5 g. or more of tyrosine to guinea pigs on an ascorbic acid-deficient diet resulted in the

abnormal excretion of homogentisic *p*-hydroxyphenylpyruvic and *p*-hydroxphenyllactic acids. Administration of 10 mg. of L-ascorbic acid daily completely corrected this abnormality. The specificity of the action of ascorbic acid was demonstrated by the fact that D-isoascorbic acid was ineffective. Subsequently, L-phenylalanine was found to be similarly implicated. Further experiments showed that feeding of *unnatural* tyrosine and phenylalanine to scorbutic guinea pigs was not accompanied, as in the case of the natural amino acids, by the excess excretion of hydroxyaromatic acids. More recently, various derivatives of phenylalanine and tyrosine have been tested and only phenylpyruvic found to behave similarly to the aromatic amino acids.

Independently and at the same time as the Sealock studies, Levine, Gordon and Marples (87) found that *premature* infants receiving diets providing 5 g. or more of protein per kg. exhibited a spontaneous defect in their metabolism of tyrosine and phenylalanine. The defect is also manifested by the excretion of L-*p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids in the urine. The defect is accentuated by feeding tyrosine and phenylalanine in pure form.

These workers subsequently found that this metabolic dysfunction was completely eradicated by the administration of 100 to 500 mg. of L-ascorbic acid to the premature infants receiving the high protein diet. Administration of D-isoascorbic acid, B-complex vitamins, crude liver extracts, and vitamin E were without effect.

Full-term infants fed similar diets did not exhibit the spontaneous defect in their metabolism of aromatic amino acids. However, the metabolic anomaly could be precipitated in full term infants by the administration of 1 g./kg. of L-tyrosine or L-phenylalanine and readily abolished by simultaneous administration of 500 mg. of L-ascorbic acid. The biochemical mechanism envolved in this aberration has been reviewed by Dr. Levine in a Harvy Lecture (88).

4. Pyridoxine and Amino Acid Metabolism

Vitamin B-6 has been intimately linked with protein metabolism in at least 3 different ways: (a) It is essential for the complete metabolism of tryptophan in the rat and swine (89). In the form of phosphorylated pyridoxal, it has been shown to function in the biological synthesis of tryptophan from indole and serine in the mold *Neurospora* (90); (b) in the form of phosphorylated pyridoxal, it is an essential prosthetic group of dicarboxylases, the enzymes which decarboxylate, tyrosine, arginine, glutamic acid, dopa, lysine, and ornithine; (c) in this form, it is also an essential prosthetic group or coenzyme for transaminases,

the enzymes which effect the transfer of amino groups of glumatic acid (and to a lesser extent of other amino acids) to keto groups to produce new amino acids.

It is clear from these metabolic relationships that the pyridoxine/protein ratio in the diet must be maintained at its optimal value to avert the symptoms of pyridoxine malnutrition as manifested by skin, blood and nerve disturbances. How these symptoms are induced by the biochemical disturbance must await more complete knowledge of the specific functions of the various amino acids. Some correlations, however, can be postulated on the basis of existing knowledge. Thus tryptophan deficiency is a causitive factor in anemia (91). Since B-6 deficiency interferes with the metabolism of tryptophan, the anemia of B-6 deficiency may be a secondary rather than a primary effect.

5. Other B-Vitamins and Protein Metabolism

Aside from metabolic relationships of the specific B-vitamins and amino acids, a number of nonspecific reactions between these two groups of substances are known to exist which effect the nutritional status of mammals. One such metabolic path is the transamination reaction referred to in the previous section. This fundamental reaction which covers the manifold interrelationships of the dicarboxylic acid system and various aspects of the metabolism of nitrogenous and non-nitrogeneous body constituents envolves the interplay of many regulatory agents, hormones, enzymes, vitamins, and ions (92). The effects of various B-vitamins insofar as they participate as prosthetic groups of enzyme components on the transamination activity (TA) have been extensively studied. Thus Kritzmann (93) has shown that the TA of tissues of *thiamin*-deficient pigeons and rats was lower than that of normal tissues; and was rapidly restored to normal by the administration of thiamin. The participation of *biotin* in this reaction was first indicated by Summerson, Lee, and Partridge (94) by their observation that the oxidation of pyruvate and lactate is regulated in part by biotin. Aspartic acid (the transamination product of oxalacetate), in bacterial culture mediums, spared appreciably the need for biotin. This is in accord with the findings of Lichstein and Umbreit (95) which demonstrated that biotin is concerned with deamination of certain amino acids including aspartic acid. These relationships, which must constitute a very vital part of our future nutritional thinking, have been set forth diagrammatically in Fig. 2.

Panthothenic acid is bound with a protein which acts as a coenzyme

for acetylation, *i.e.*, formation of acetylcholine or detoxification of certain drugs.

The metabolic relationship of the newly discovered vitamin B-12 and protein metabolism has not as yet been completely assessed (96). Even at this early date, however, it is clear that the biosynthesis of a number of biologically important proteins is influenced by this substance. Its effectiveness in the treatment of pernicious anemia appears to be related to its biocatalytic action of nucleoproteins and more specifically with the

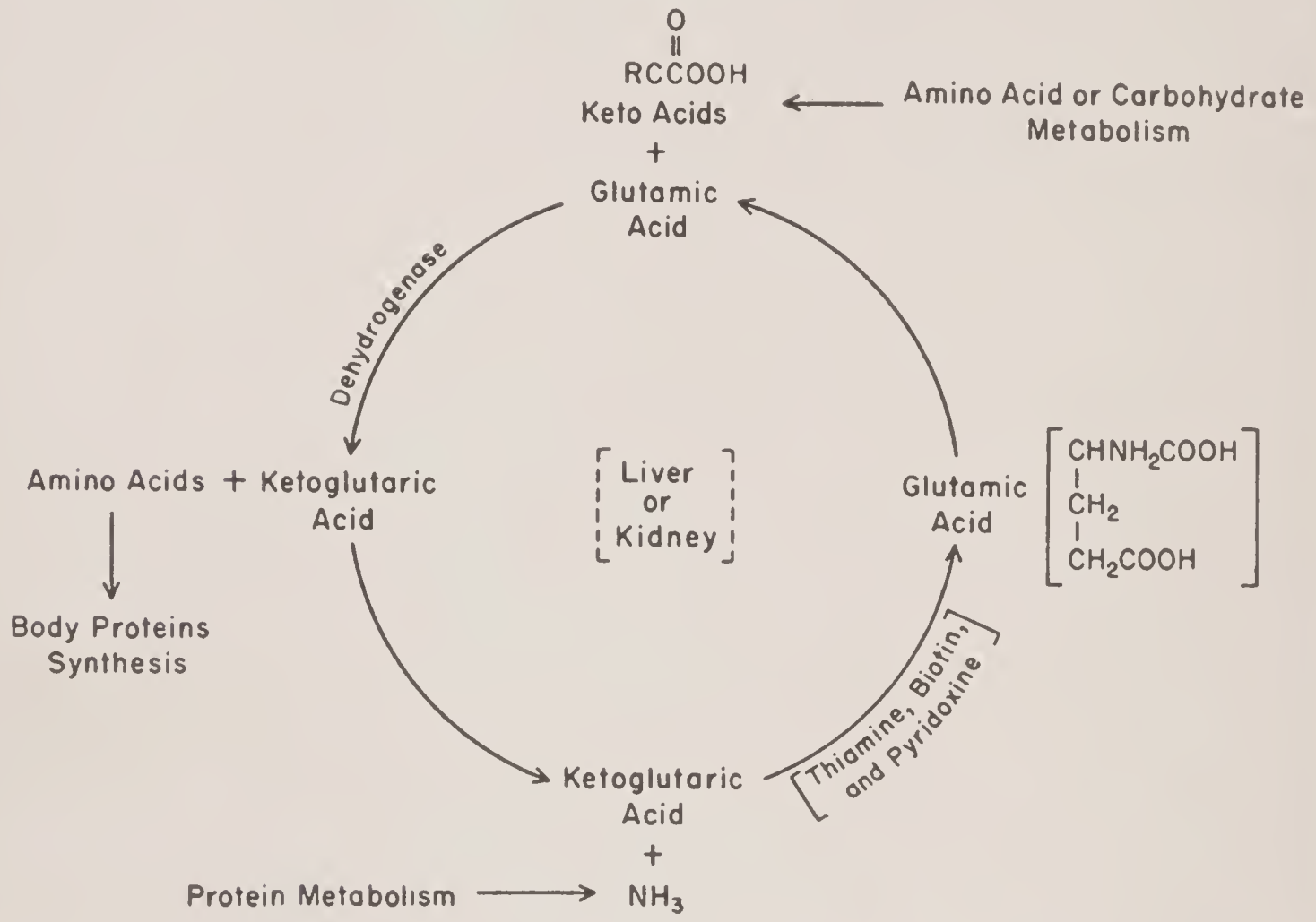

Fig. 2. Diagrammatic representation of the biological synthesis of amino acids by transamination.

transformation of thymine to thymidine (97). However, because of the large amounts of thymidine required to replace B-12, the two are not considered identical. Rose (40) has suggested that poor biological value of synthetic diets employed by his group for the determination of the amino acid needs of the adult may be due in part to a B-12 deficiency.

IX. Relation of Hormones, Proteins, and Amino Acids

There exists no sharp dividing line between vitamins, *i.e.*, *exogenous biocatalysts* taken with the food and hormones and enzymes, *i.e.*, *endogenous biocatalysts* produced within the body. Ascorbic acid, shown above to be concerned with the metabolism of tyrosine and phenylalanine,

is an exogenous catalyst, a vitamin in man, monkey, and guinea pig, but an endogenous catalyst in the rat and dogs. The status of mice, rabbits, swine, and cattle in this regard is not clear.

1. Biosynthesis of Hormones

According to their chemical nature, the known hormones can be classified into 3 groups which are metabolically related to amino acids and proteins in different ways.

a. Phenolic Derivatives. Adrenalin, the hormone of the adrenal medulla (98) and thyroxin, the hormone of the thyroid are derived from tyrosine or phenylalanine. While both adrenalin and thyroxin are metabolic (oxidation) catalysts, the time relations of their effects are different. The influence of the thyroid on metabolism is a long-range steady type. From a nutritional aspect, factors affecting the synthesis of thyroxin, *i.e.*, deficiency of I_2 or phenolic derivatives, could be expected to have a profound and measurable effect on the nutritional state.

b. Proteins. To this group belong the hormones of the hypophysis which include two gonadotropins, thyrotropin, adrenocorticotropin, lactogenic hormones and growth promoting factors from the anterior lobe; and the vasopressor, oxytocic and melanic expanding principles of the posterior lobe. The hormone of the pancreas, and the hormone of the parathyroid (parathormone) also belong in this group. These protein hormones, in addition to requiring all of the amino acids for their formation, also require in some instances greater quantities of specific amino acids than most tissue (99).

c. Steroids. To the steroid family belong the hormones isolated from the ovarian, corpus luteum, testis and adrenal cortex. In the light of recent developments, the possibility that the amino acids may serve as source substances for these hormones does not appear to be as remote as was thought heretofore. This biochemical linkage received its first experimental hope in Bloch's recent demonstration that various active steroids are derived biosynthetically from cholesterol (100) and the convincing experiments of Rittenberg (101) which show that cholesterol is synthesized from acetic acid in the mammal. If we permit the reasonable speculation that acetic acid, pyruvic acid, and other organic acids are derived in part by deaminization of aliphatic amino acids or degradation of tyrosine (102), then it is apparent from the foregoing that amino acid deficiencies affect the physiological integrity of the mammalian organism; not only by a reduced biosynthesis of proteins of the supporting structure, but also by reduction in hormonal synthesis. Direct evidence is lacking for this conclusion. However, the pathological lesions

produced by dietary deficiencies of tryptophan (103), valine (104), and other amino acids (105) cannot be wholly explained by the simple reduction of supportative tissue proteins.

The suboptimal growth observed in mice, rats, and infants fed diets containing acid protein hydrolysate or mixtures of purified amino acids as contrasted with the rate of growth obtained with diets containing whole proteins or enzymatic digest of protein, might well be due to the lack of some essential peptide of a hormonal nature which is destroyed by acid treatment.

Recent reports by Li and associates on the chemical nature of ACTH indicates that peptide moieties of hormonal protein bear considerable biocatalytic activity (106). This protein (ACTH) which may be isolated from either sheep or pig or pituitary glands in pure form has a molecular weight of 20,000 and an isoelectric point at pH 4.70. The hormone contains 15.65 N, 2.30 S, the S content being accounted for by 1.93 methionine and 7.19 per cent cystine. The reaction of ACTH with ketone, HNO_2, formaldehyde and iodine indicate that both the free amino and tyrosine groups are essential for the hormonal action. Fragments obtained by pepsin digestion or 6M HCl hydrolysis containing an average 8 peptide chains, which are not precipitable by TAA and dialysable, exhibit as much biological activity as the parent substance. From these experiments, it may be concluded that a peptide fragment of low molecular weight is responsible for the adrenocorticotropic potency.

The metabolic importance of this finding, and the new role of peptides in human physiology, can be readily appreciated from the following statement of Dr. Long (107): "In other words as far as can be determined at the present time all circumstances that enhance the secretion of the adrenal cortex can only do so by first activating the anterior lobe of the pituitary, in consequence of which the required quantity of A.C.T.H. is released." It seems possible that during the assimilation of protein prefabricated peptide units having such activity, might become directly available to the body. If the diet supplies only free amino acids, then the hormone has to be produced *de novo*. The delay so incurred, however small, assumes great importance because of the regulatory functions of this product on the metabolic reaction of the organism which will be now discussed.

2. Anabolic and Catabolic Reactions

Thyroid administration or hyperthyroidism accelerates all metabolic processes which increases the need for almost all nutrients, proteins,

minerals, and vitamins. If the caloric and protein needs are satisfied, but the vitamin needs are not; hyperthyroidism obviously will be associated with and show symptoms of hypovitaminosis. The cardiac symptoms (bradycardia and tachycardia) and hepatic damage (particularly low liver glycogen) associated with hyperthyroidism, are confused by the cardiac and hepatic syndrome associated with B-vitamin deficiency, particularly of thiamin.

It is human to assume, as did Mossu in 1899, that if some thyroid is essential for normal growth, a little more thyroid would result in supernormal growth and he indeed reported acceleration of growth rates in normal dogs by feeding small amounts of thyroid tissues. However, the growth in the thyroid-treated animals stopped earlier than in the controls and mature size was slightly subnormal (108). There is a striking species variability in reaction to thyroid administration. Thus, the growth rate of mice is increased by a certain thyroid dosage (.19 mg. fresh thyroid *per os* daily or by daily injection of 0.01 to 0.03 mg. crystalline thyroxin) (109). The mature size was not changed; only the maturation rate or early growth rate was accelerated. Koger and Turner could not accelerate the growth of rats, guinea pigs, or rabbits by any thyroid dosage. The growth rate of the smaller breeds of domestic fowl is apparently accelerated by thyroid administration but not of the larger. The growth of some children is accelerated by feeding about 65 mg. of desiccated thyroid per day. The individual variations in response, however, cast doubt on the significance of this finding for the practical acceleration of the growth process (110).

It is generally accepted, however, that thyroid medication is useful in some cases of human sterility. An experiment by the author suggests that the effectiveness of this therapy may be increased by arginine supplementation of the diet, the amino acid which constitutes approximately 80 per cent of sperm protamine (Fig. 3).

Observations by Turner (111) on two-year old Leghorn hens, indicate greatly increased egg production over several months associated with thyroid active material. There is also considerable literature (112) indicating that milk production is increased up to 50 per cent, at least for short periods on the declining segment of the lactation curve, by feeding thyroidactive material (15 g. iodinated casein daily). However, the ascorbic acid content (no data on other vitamins) in the milk is reduced (113). This treatment accelerates the rate of senescence (114), but this is without agricultural significance since animals are treated only during the year before slaughter. The availability of inexpensive iodinated casein and peanut protein makes this treatment of farm animals economically feasible.

Of the nitrogenous hormones, the metabolic effects of the previously described ACTH merit some attention. This substance is a vigorous catabolic agent which appears to have numerous therapeutic attributes. It has been shown by a number of investigators that this activity is expressed by a urinary loss of N, P, Ca, K, Na, and Cl, which follows immediately the administration of ACTH. These changes are accompanied by a retention of fluids and an increase in body weight (115).

The effect of ACTH on the circulatory antibodies and other elements in man have been studied by Venning, de Vries, and Herbert (116).

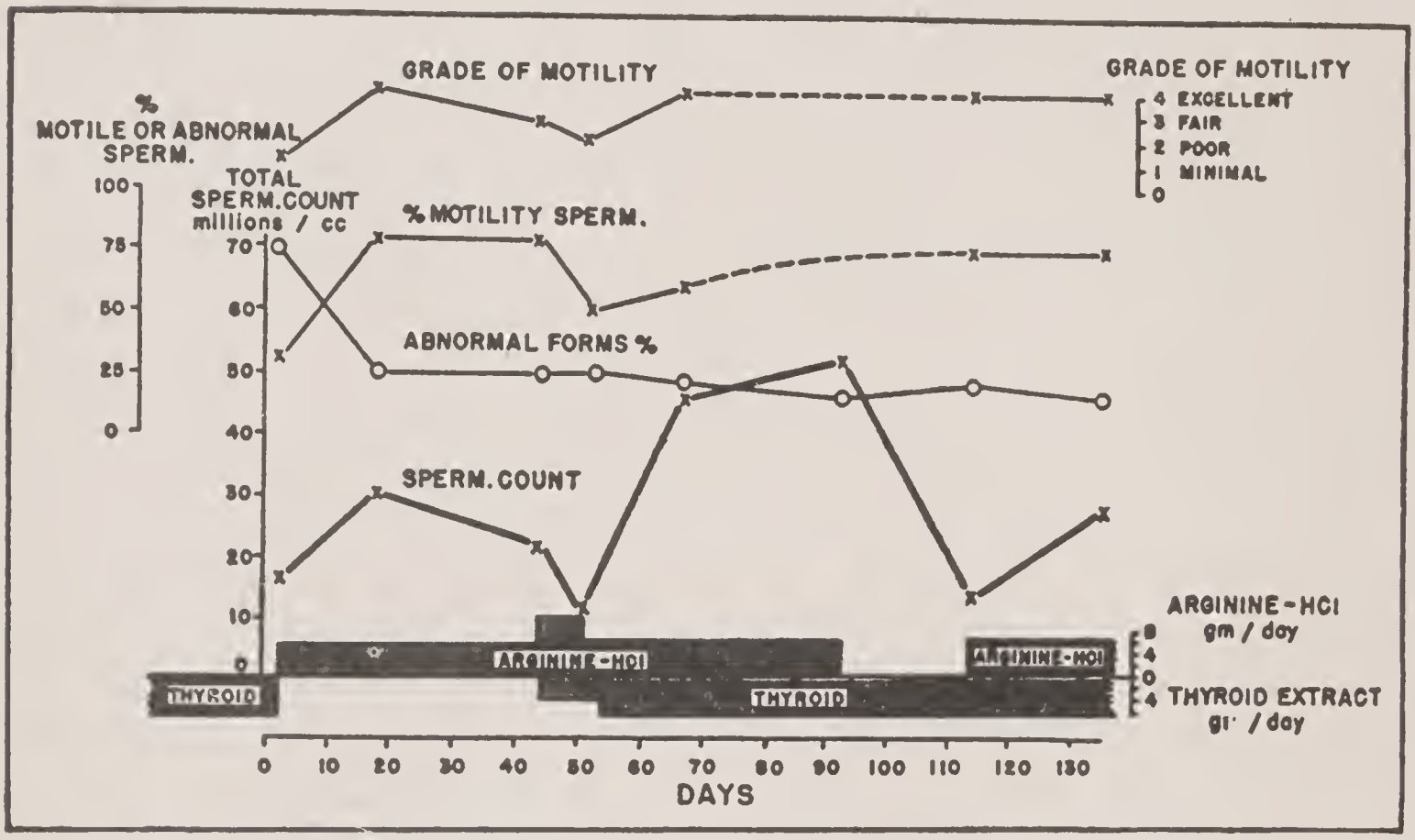

Fig. 3. Effect of arginine supplement on spermatogenesis of patient (M.R., 30 yrs.) with idiopathic hypospermia receiving thyroid therapy.

Their results indicate that ACTH administration does not accelerate antibody response in healthy or diseased humans. It was also noted that ACTH therapy caused a rapid blood dilution, indicated by the simultaneous and parallel decrease in hematocrit and plasma proteins. In one experiment, a blood sample taken 30 minutes following intramuscular injection of 40 mg. of ACTH, the plasma proteins fell from 8.35 to 7.8; and the hematocrit from 45.9 to 43 per cent. This account of the various aspects of protein metabolism could not be justly concluded without some reference to the profound effects of the steroid hormones on the metabolic processes involving N utilization. Numerous reports are available on the anabolic activity of the androgens in rats and man. Kochakian (117) has compared the protein anabolic properties of various steroids in the adult castrated rat, and found that

testosterone propionate is the most efficient of all of the steroids studied.

Because of the effect of androgens on the metabolism of N, P, S, and other elements essential to tissue building, these hormones have been employed as an adjunct in the care of premature infants. Shelton and his associates (118) have found that androgens distinctly shortened the time required to regain normal birth weight. In a group of babies weighing 1000 to 1500 g., the controls required an average of 14.7 days to regain the birthweight; their infants on methyl testosterone required 9.0 days and those on testosterone propionate only 7.5 days.

Brown and coworkers (119) have made a 10-year study on the effects of testosterone compounds on growth in man. This survey deals with the findings on 26 underdeveloped children, ranging in age from 12 to 17 years and who were below their expected height. Growth increments were observed in all instances which ceased with the withdrawal of the treatment. Wilkins and associates (120) have reported on the effects of testosterone propionate and methyl testosterone in sexually immature dwarfs, and noted marked improvements in body weight and N-balance with the administration of these steroids. A more comprehensive study was reported by Harding in 1948 (121) which covered the treatment of 58 boys between the age of 7 and 15 with oral methyl testosterone. The rate of growth of this group was increased, the average being 3.2 inches per year, while control children grew only 2.3 inches per year.

The anabolic action of androgens is not confined to the growth periods alone, but has been observed repeatedly in convalescence from physiological shock; a period marked by a profound negative N-balance which some believe cannot be influenced by a simple increase in protein content of the diet (122, 123). Recent observations in our laboratory indicate that N-balance and weight of individuals aged 60 to 70 years can be dramatically improved within a week by the daily administration of intramuscularly injected testosterone propionate, 10 mg. (124).

It must be pointed out that androgens do not manufacture nitrogen, but only aid in storing it. It is important, therefore, that the protein in the diet be adequate, and if this cannot be achieved by the oral route, then amino acids must be given parenterally to cover these needs. This raises the question of the relative effectiveness of various proteins or amino acid mixtures which may be used to support the anabolic reactions. Conceivably, a certain mixture of amino acids could support a greater anabolic effect than others with a given quantity of androgen. The determination of the composition of such mixtures is of practical value, since in this manner, the objectionable virilism which accompanies

the anabolic reactions of androgens might be reduced to a minimum by the use of smaller dosages.

An effort in this direction was made by our group at Johns Hopkins in 1943. In order to ascertain the effect of androgens on the S-amino acid needs, we compared the urinary output of S- and N-metabolites of a cretin treated with testosterone propionate. The results of this experiment are collected in Table X.

TABLE X

The Effect of Testosterone Propionate and Thyroid on Nitrogen, Sulfur, and Amino Acid Metabolism

Patient: SS, male; 13.5 kg.; 4.5 years
Diagnosis: Hypothyroid cretin; "height age" 19 mos.; "bone age" 3 mos.
Food intake per day: 1200 calories; protein 30 to 35 gm.

(All results given as daily average of 5-day period)

Period	1	2	3
Date	10/28—11/1	11/13—11/17	11/24—11/30
Treatment	none	T-P:[a] 20mg./day	Thyroid: 2g./day
Urine volume, cc.	1200	1100	1400
Total urine N, g.	4.94	2.88	7.70
Methionine, mg.	137	206	233
Cystine, mg.	40	24	36
Indican, mg.	4.5	3.2	15.2
SO_4—S, g.	0.90	0.58	1.43
Total urine S, g.	0.30	0.19	0.48
Creatine, mg.	102	98	195
Creatinine, mg.	464	432	640
Body weight change, gm.	0	700	—450

[a] T-P = Testosterone propionate, (Ciba-Perandren).

It is to be noted that the output of methionine was increased in the face of a decreased total S-output during testosterone treatment, which suggests a decreased need or utilization of this amino acid under these conditions. Attention is called to the effect of thyroid therapy on these same metabolites and especially the continued high methionine output. The biochemical interpretation of these findings must await further studies of this type which we have recently undertaken in this laboratory.

X. Summary

This discussion of the factors affecting the nitrogenous needs of mammals, because of the very nature of the subject, had to be extensive and therefore not readily susceptible to summary. However, some

thoughts which comprise the underlying thesis of the presentation are reassembled here for emphasis.

With the continuing discovery of new essential food factors and biochemical interdependencies of various food elements, it is becoming ever increasingly clear to nutritionists that measurements made with synthetic diets may be shortly invalidated by deficiencies of previously unknown essential nutrients or nutrile relationships. It is of course recognized that highly purified diets often provide the only experimental approach to many nutritional problems, especially those for which the identity of new food components are being sought.

When it is desired to measure the quantitative requirements of a specific nutrient, however, then the number of unknown variables of the diet should be reduced to a minimum. Furthermore, if the measured requirements of an amino acid is to have any practical significance whatsoever, it seems logical that experimental matters be so arranged that the quantitative and qualitative composition of the diet simulate as closely as possible the natural foods in respect to total calories, distribution of calories derived from fats, carbohydrates and proteins, vitamin content and relative quantities of essential and unessential amino acids. On this basis, which fully recognizes important biochemical mechanisms, such as the protein sparing action of carbohydrates, the essential amino acid sparing action of unessential amino acids, proper distribution of specific dynamic action, and biocatalyst amino acid interrelation ships, it appears reasonable to recommend the acceptance of amino acid and protein requirement values secured with the essentially normal diets.

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Index

A

Acetic acid, 141
ACTH, 142-144
Adrenalin, 140
Adult human requirements, 19-20, 48, 126-127, 129
Alanine, 64, 120, 125
Albumin, blood, 97, 100
  egg, 80, 86, 100
Albumin: globulin ratios, 100, 102
Amino acid imbalance, 58, 90
Amino acids,
  biosynthesis by transamination, 61, 140
  deficiency effects, 88-90
  dietary value of mixtures, Vuj, 82, 83
  "essential," 59, 64
  growth pattern, 45, 127
  hormones, relation to, 140
  maintenance pattern, 45, 127
  "non-essential," 59
  requirements, qualitative,
    in chicks, 118
    in dogs, 121
    in man, 121
    in mice, 118
    in rats, 118, 120
  requirements, quantitative,
    of infants, 122
    of human adults, 19, 48, 124
    of immature rats, 122
    of adult rats, 19, 45, 48, 57
  utilization of *d*-amino acids, 18, 67-70
    by dogs, 67
    by man, 67
    by rats, 67
Ammonium salts, 3, 59, 60, 62
  dietary value, 65-66
Anabolism, of proteins, 142
Anemia, 139
Animal tissues, composition of, 14
Antibody production, 132
Arginine, 14, 16, 19
  dietary value, 6, 120, 122, 125
  in hypospermia, 144
  role of, 62
Ascorbic acid,
  in aromatic amino acid metabolism, 137
Aspartic acid, 62, 64, 120, 125
  transamination and role of biotin, in, 139

B

Beef liver, 86
Beef muscle, 4, 5, 56, 125
Blood proteins, 13, 14, 23, 125
  factors effecting formation, 23, 80, 102-107

C

Carbohydrates,
  protein sparing action, 134
  relation to amino acid metabolism, 134, 135
Casein, 77, 86, 95, 100
  amino acid content, 125
  hydrolysates, biological value, 4, 5, 42, 56, 100, 105
  hydrolysate fraction, 106, 112
  relation to blood protein formation, 102
Catabolism, 24, 142
Chick, requirements, 16, 118, 120
Cholesterol, 141
Choline, 136
Convalescence, 128
Connective tissues, 14
Cystine, 5, 10, 14, 19, 95
  methionine sparing effect, 124

D

Detoxication, 95
Dextrimaltose, 135
Dicarboxylic acids, 119
Diet, protein free, 100, 109
Dogs, 86, 97-100, 117
Dynamic state, of body protein, 24, 39

E

Egg albumin, 4, 5, 56, 86, 100
Egg proteins, 4, 5, 39, 56, 81, 100
Enzymes, 94, 111, 140
Epinephrine, 141

F

Fats, in metabolism, 135
Fibrin hydrolysate, 42
Fructose, 135

G

Galactose, 134
Galactosemia, 134
Gelatin, 3, 77, 86
Globin, beef, 125
Globulins, serum,
  formation, 100-103
Glutamic acid, 119, 120, 125
  in transamination, 60, 64
Gluten, 56
Glycine, 64
Gonadal, growth, 108
Growth, 143, 145
  amino acid requirements, 13, 127
  amino acid pattern, 45, 127

H

Hexone bases, 119
Histidine, 6, 14, 16, 19, 45, 48
Hormones, 94, 110, 112, 140-146
Hydrolysates, liquid, 49-50
Hydroxyproline, 120, 125

I

Infants, 122
  amino acid requirements, 119, 127
Intravenous alimentation, 40
Ion-exchange resins, 119
Isoleucine 6, 14, 16, 19, 43, 45, 48, 127
Isotopes, 3, 22, 26, 60, 127

K

Keratins, 9, 12, 14
  synthesis, 10, 23, 27
α-Keto acids, 61, 70

L

Lactalbumin, 95, 100
  composition, 125
  dietary value, 77, 86
  in formation of blood proteins, 103
  hydrolysates, 100, 105
Lactation, 95
Lactose, 135
Leucine, 6, 14, 16, 19, 45
  requirements of different species, 120
Liver proteins, 51-52, 78-82, 107-108
Lysine, 5, 14, 16, 19, 28
  dietary values, 6, 8, 9
  metabolism, 121, 133
  requirement differences, 8, 48, 95, 120, 122, 127
Longevity, 131

M

Maintenance of body weight,
  requirements for, 45
Malnutrition,
  effect on dietary requirements, 130
Meat proteins, 125
Metabolic pool, 25
Metabolism,
  acceleration, 143
  basal, 7, 129
  endogenous, 22, 129
  exogenous, 21, 24
Methionine, 6, 10, 14, 16, 54
  lipotropic activity, 19, 45, 48
  requirements for, 8, 123, 124
Methyl group, 136
Milk,
  allergy in young animals, 117
  composition and rate of growth, 116
  dietary value of proteins, 125
Muscle proteins, 15, 125
  composition, 13, 14

N

Neoplasms, 123, 128
Nicotinic acid, 137
Nitrogen balance, 85-91
  effect of N intake level on, 130, 131
  methods, 36-39
  significance 3, 18, 133

Nitrogen minimum, 39, 128, 129
Nitrogen retention, 88, 89
Norleucine, 121

P

Peanut flour, 4, 5, 56
Peptides, availability, 92
Phenylalanine, 6, 14, 16
  requirements for, 19, 45, 48, 124, 133
Plasma proteins,
  depletion, 96
  regeneration, 104
Plasmaphoresis, 97, 98
Polypeptides, 96, 111
Proline, 120
Proteins,
  amino acid composition, 14, 125
  body, 14, 17
  biological value, 4, 35, 56, 80, 100
  deprivation, 42
  dietary requirements, 10, 116
    for gonadal growth, 108
    for various ages and states, 117
  hydrolysates,
    dietary value, 46, 47, 85
    intravenous alimentation, 41, 42
    relation to blood proteins, 101
  metabolism theory, 20, 26
  regeneration, 43, 56, 57
  reserve stores, 22, 98, 112, 130
Pyridoxine, 138

R

Rat,
  repletion methods, 44, 77, 78-82, 98-99
    critique, 51-54
    minima, 57
  requirements, 16, 19, 48, 90

S

Serine, 120, 125
Soya meal, 81, 86, 110
Specific dynamic action, 136
Spermatogenesis, 143
Starvation, 129, 130
Steroids, 141
Strepogenin, 96, 112, 133

T

Thiamine, 140
Testosterone, 145, 146
Threonine, 6, 14, 16, 19, 45, 49, 120, 125
Thyroid, 143, 146
Thyroxine, 143
Transamination reactions, 62, 139, 140
Tryptophane, 2, 6, 14, 16, 19, 43, 45, 48, 49, 95, 120, 125
  deficiency, 50, 142
  pellagra treatment with, 137
  utilization of D-form, 67
Tyrosine, 14, 19, 120, 125, 133, 137, 141

U

Urea, 3, 61, 71, 128

V

Valine, 6, 14, 16, 19, 45, 120, 125
Vitamins, 136-140

W

Water needs, 49
Wheat proteins, 56, 100, 125
Wool, 11

Y

Yeast, 80, 86

Z

Zein, 2, 54

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